A Family of Single Copy repABC-Type Shuttle Vectors Stably Maintained in the Alpha-Proteobacterium Sinorhizobium meliloti

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Supporting Information

ABSTRACT: A considerable share of bacterial species maintains segmented genomes. Plant symbiotic α-proteobacterial rhizobia contain up to six repABC-type replicons in addition to the primary chromosome. These low or unit-copy replicons, classified as secondary chromosomes, chromids, or megaplasmids, are exclusively found in α-proteobacteria. Replication and faithful partitioning of these replicons to the daughter cells is mediated by the repABC region. The importance of α-rhizobial symbiotic nitrogen fixation for sustainable agriculture and Agrobacterium-mediated plant transformation as a tool in plant sciences has increasingly moved biological engineering of these organisms into focus. Plasmids are ideal DNA-carrying vectors for these engineering efforts. On the basis of repABC regions collected from α-rhizobial secondary replicons, and origins of replication derived from traditional cloning vectors, we devised the versatile family of pABC shuttle vectors propagating in Sinorhizobium meliloti, related members of the Rhizobiales, and Escherichia coli. A modular plasmid library providing the essential parts for pABC vector assembly was founded. The standardized design of these vectors involves five basic modules: (1) repABC cassette, (2) plasmid-derived origin of replication, (3) RK2/RP4 mobilization site (optional), (4) antibiotic resistance gene, and (5) multiple cloning site flanked by transcription terminators. In S. meliloti, pABC vectors showed high propagation stability and unit-copy number. We demonstrated stable coexistence of three pABC vectors in addition to the two indigenous megaplasmids in S. meliloti, suggesting combinability of multiple compatible pABC plasmids. We further devised an in vivo cloning strategy involving Cre/loxP-mediated translocation of large DNA fragments to an autonomously replicating repABC-based vector, followed by conjugation-mediated transfer either to compatible rhizobia or E. coli.

KEYWORDS: plasmid cloning vehicle, Cre/loxP, repABC, in vivo cloning, Sinorhizobium, Rhizobiales

Plasmid vectors are valuable tools in genetic engineering and synthetic biology. Most well established cloning vectors have specifically been selected and designed to be applied in Escherichia coli. Plasmids with a broader host range that can be transferred between different microorganisms and function in diverse genomic backgrounds represent a natural source of orthogonal DNA elements autonomously replicating in bacteria. While medium to high copy plasmids are usually randomly distributed during cell division, low copy plasmids employ a segregation mechanism to assort plasmid copies to each daughter cell. Plasmids can be considered as ideal DNA-carrying vectors with respect to the ease of plasmid engineering, modularization, and standardization. Yet, variations in copy number and propagation stability, as well as limited cloning capacity and combinability are unfavorable properties of plasmid vectors. Unreliable plasmid propagation has to be overcome by selection pressure that may negatively affect cell growth and physiology. The main bacterial chromosome has been used as vector for assembly and stable propagation of megasize DNA, and also engineered or synthetic chromosomes potentially offer high propagation stability and cloning capacity.

Several members of the Rhizobiales belonging to the class of α-proteobacteria are best known for their ability to establish a nitrogen-fixing symbiosis with legumes or as plant pathogens of the genus Agrobacterium causing crown gall tumors on many vascular plants. Advances in synthetic biology have increasingly moved utilization of genetic resources and processes underlying the microbe-plant interactions of these α-proteobacteria into focus. This includes the high value of symbiotic nitrogen fixation for sustainable agriculture, but also...
| strains/plasmids                  | description                                                                 | refs  |
|----------------------------------|-----------------------------------------------------------------------------|-------|
| **S. meliloti**                   |                                                                             |       |
| Rm1021                           | wild type strain                                                           | 22    |
| ΔhsdR                            | Rm1021 strain with hsdR deletion                                            | 45    |
| SmCreΔhsdR                       | Rm1021 cre expression strain with hsdR deletion, tauX:: cre-tetRA (Tc')      | 45    |
| SmA18                            | strain 2011 cured of megaplasmid pSymA                                     | 79    |
| JDSm106                          | Rm1021 bearing tetR-YFP-mCh-parB<sub>Ty</sub> (pJD120) under control of PltauAB | this study |
| MWSm8                            | Rm1021 bearing pMW1 genomically integrated (Km')                            | this study |
| MWSm37                           | ΔhsdR bearing pMW28 for constitutive egfp expression (Km')                  | this study |
| SmCre<sub>exo-IN</sub>           | SmCreΔhsdR bearing pIso and P<sub>min2</sub>-loxR-aacC1 integrated up- and downstream of the exo gene cluster, respectively (Km', Gm') | this study |
| SmCre<sub>exo-OUT</sub>          | SmCreΔhsdR carrying pIsLo-exo isolated from pSymB (IPTG dependent) (Km', Spec') | this study |
| **A. tumefaciens**                |                                                                             |       |
| CS8                              | wild type strain                                                           | 24, 25|
| CS8<sub>exoB</sub>               | exhibiting a CFGE-mediated inactivation of exoB (Gm')                      | 45    |
| exoB/plso-exo                    | CS8 exoB carrying pIsLo-exo (IPTG dependent) (Gm', Km', Spec')              | this study |
| **R. etli**                      |                                                                             |       |
| CFN42                            | wild type strain                                                           | 80    |
| WSM419                           | wild type strain                                                           | 81    |
| CC169                            | wild type strain                                                           | 82    |
| **S. medicae**                   |                                                                             |       |
| **R. leguminosarum**             |                                                                             |       |
| MAF303099                        | wild type strain                                                           | 21    |
| **M. extorquens**                |                                                                             |       |
| AM1 Δcel                         | strain AM1 deficient in cellulose production                                | 85    |
| **C. crescentus**                |                                                                             |       |
| CB15 N                           | wild type strain                                                           | 86    |
| **E. coli**                      |                                                                             |       |
| DH5α                             | used for cloning procedures and plasmid extraction                          | 87    |
| S17–1                            | used for conjugation with *S. meliloti*                                     | 88    |
| XL1-Blue                         | used as recipient of pRK2013 (Tc')                                        | Stratagene |
| XL1Blue-pRK2013                  | used for triparental matings (Tc', Km')                                    | this study |
| AR3219                           | used as Clm resistant recipient of pISO-exo (Clm')                         | 89    |
| **Plasmids**                     |                                                                             |       |
| pK/G18mob2                       | suicide vector carrying replication origin pMB1                            | 39    |
| pK18mobscB                       | suicide vector carrying sacB for sucrose selection                          | 39    |
| pSRK-Gm/Km                       | broad-host-range expression vector                                         | 90    |
| pXG-10                           | carrying replication origin pSC101* (derivative of pSC101 exhibiting reduced copy number in *E. coli*) | 91    |
| pACYC184                         | carrying replication origin p1SA                                           | 92    |
| pPHU2331                         | broad-host-range expression vector                                         | 93    |
| pRR2013                          | helper plasmid for mobilization of nonself-transmissible plasmids           | 94    |
| pLAU44                           | pUC18 derive carrying tet operator (tetO) cassettes                         | 57    |
| pART                             | artificial mini-replicon based on a pSymA derived repA2B2C2 cassette       | 58    |
| pK18mob2-repABC<sub>pSymA</sub>  | pK18mob2 carrying a pSymA derived repA2B2C2 cassette                       | this work |
| p42b_p                           | pK18mob2 carrying repABC<sub>p42b</sub> based oriV (R. etli)               | this work |
| p42d_p                           | pK18mob2 carrying repABC<sub>p42d</sub> based oriV (R. etli)               | this work |
| p42e_s                           | pK18mob2 carrying a truncated repABC region derived from p42e (R. etli)    | this work |
| p42l1_p                          | pK18mob2 carrying repABC<sub>p42l1</sub> based oriV (R. etli)              | this work |
| p42l2_p                          | pK18mob2 carrying repABC<sub>p42l2</sub> based oriV (R. etli)              | this work |
| pMla_s                           | pK18mob2 carrying a truncated repABC region derived from pMla (M. loti)     | this work |
| pMlb_p                           | pK18mob2 carrying repABC<sub>pMlb</sub> based oriV (M. loti)               | this work |
| pSMED01_p                        | pK18mob2 carrying repABC<sub>SMED01</sub> based oriV (S. medicae)          | this work |
| pSMED02_p                        | pK18mob2 carrying repABC<sub>SMED02</sub> based oriV (S. medicae)          | this work |
| pNGR234b_p                       | pK18mob2 carrying repABC<sub>NGR234b</sub> based oriV (S. fredii)         | this work |
| pTi_p                            | pK18mob2 carrying repABC<sub>Ti</sub> based oriV (A. tumefaciens)          | this work |
| pMW1                             | integrative pK18mob2 derivative conferring Km'                              | this work |
| pMW28                            | integrative pK18mob2 derivative carrying Pnm2-egfp                          | this work |
| pJD120                           | pK18mobscB derivative, integration of tetR-YFP-mCh-parB<sub>Ty</sub> under control of PltauAB | this work |
Table 1. continued

| Plasmids                      | description                                                                 | refs       |
|-------------------------------|-----------------------------------------------------------------------------|------------|
| pJD211                        | pK18mob2 derivative carrying Pmin2-loxR-aacC1                               | this work  |
| pPHU231-egfp                  | pPHU231 derivative carrying a constitutive egfp cassette                    | this work  |
| pABCa-egfp                    | pABCa carrying a constitutive egfp cassette                                 | this work  |
| pABCa-egfp-expR               | pABCa carrying constitutively expressed egfp-expR                           | this work  |
| pABCa-tetO                    | pABCa carrying a tetO array                                                 | this work  |
| pABCb-egfp                    | pABCb carrying a constitutive egfp cassette                                 | this work  |
| pABCb-egfp-expR               | pABCb carrying constitutively expressed egfp-expR                           | this work  |
| pABCc-egfp-expR               | pABCc carrying constitutively expressed egfp-expR                           | this work  |
| pABCc-parSf                  | pABCc carrying parSf                                                       | this work  |
| pABCc-mob-egfp                | pABCc-mob carrying a constitutive egfp cassette                             | this work  |
| pABC1-egfp                    | pABC1 carrying a constitutive egfp cassette                                 | this work  |
| pABC1-egfp-expR               | pABC1 carrying constitutively expressed egfp-expR                           | this work  |
| pABC1-egfp_f                  | pABC1 carrying a forwardly integrated SD-egfp fragment                      | this work  |
| pABC1-egfp_r                  | pABC1 carrying a reversely integrated SD-egfp fragment                      | this work  |
| pABC1-T2-egfp_f               | pABC1-T2 carrying a forwardly integrated SD-egfp fragment                   | this work  |
| pABC1-T2-egfp_r               | pABC1-T2 carrying a reversely integrated SD-egfp fragment                   | this work  |
| pABC1-T3-egfp_f               | pABC1-T3 carrying a forwardly integrated SD-egfp fragment                   | this work  |
| pABC1-T3-egfp_r               | pABC1-T3 carrying a reversely integrated SD-egfp fragment                   | this work  |
| pABC2-egfp                    | pABC2 carrying a constitutive egfp cassette                                 | this work  |
| pABC2-egfp-expR               | pABC2 carrying constitutively expressed egfp-expR                           | this work  |
| pABC3-egfp                    | pABC3 carrying a constitutive egfp cassette                                 | this work  |
| pABC4-egfp                    | pABC4 carrying a constitutive egfp cassette                                 | this work  |
| pABC4-egfp-expR               | pABC4 carrying constitutively expressed egfp-expR                           | this work  |
| pABCb-lacI                    | pABCb carrying lacI for constitutive LacI expression                       | this work  |
| pMb _lacO                     | pK18mob2 carrying repABC_plab bearing a lacO cassette at the predicted transcription start site | this work  |
| pso_                            | pABC cloning vector integrating upstream of the exo gene cluster on pSymB  | this work  |
| pso-exo                        | isolated ABC cloning vector carrying the exo gene cluster (replicative upon IPTG supplementation) | this work  |
| pABC Library Plasmids Carrying Module Parts | pLoriVSm derivative, unloaded library vector for oriVSm parts    | this work  |
| pLoriVSm                      | pK18mob2 derivative, unloaded library vector for synTer-MCS parts          | this work  |
| pLoriT-MCS                    | pK18mob2 derivative, unloaded library vector for oriT parts                | this work  |
| pLoriT                         | pK18mob2 derivative, unloaded library vector for AR parts                  | this work  |
| pLoriV-42b                    | pLoriVSm derivative providing the oriV42b region (R. etli)                 | this work  |
| pLoriV-42d                    | pLoriVSm derivative providing the oriV42d region (R. etli)                 | this work  |
| pLoriV-42e                    | pLoriVSm derivative providing the oriV42e region (R. etli)                 | this work  |
| pLoriV-42f                    | pLoriVSm derivative providing the repA1B1C1 based oriV42f region (R. etli) | this work  |
| pLoriV-42r                    | pLoriVSm derivative providing the repA2B2C2 based oriV42r region (R. etli) | this work  |
| pLoriV-Mla                    | pLoriVSm derivative providing the oriVm1a region (M. loti)                 | this work  |
| pLoriV-Mlb                    | pLoriVSm derivative providing the oriVm1b region (M. loti)                 | this work  |
| pLoriV-Ti                     | pLoriVSm derivative providing the oriV42T region (A. tumefaciens)           | this work  |
| pLoriV-T1                     | pLoriVSm derivative providing synTer1-MCS                                  | this work  |
| pLoriV-T2                     | pLoriVSm derivative providing synTer2-MCS                                  | this work  |
| pLoriV-T3                     | pLoriVSm derivative providing synTer3-MCS                                  | this work  |
| pLoriV-T-1-lex                 | pLoriVSm derivative providing synTer1-MCS-lex                               | this work  |
| pLoriV-T-2-lex                 | pLoriVSm derivative providing synTer2-MCS-lex                               | this work  |
| pLoriV-T-3-lex                 | pLoriVSm derivative providing synTer3-MCS-lex                               | this work  |
| pLoriT-1                      | pLoriT derivative providing a mob site                                     | this work  |
| pLoriT-1-lex                   | pLoriT derivative providing mob-lexlR                                      | this work  |
| pLoriT-1-1exT                  | pLoriT derivative providing mob-lexlR-expR-T2                              | this work  |
| pLAR-Km                        | pLAR derivative providing a kanamycin resistance cassette                  | this work  |
| pLAR-Spec                      | pLAR derivative providing a spectinomycin resistance cassette              | this work  |
| pLAR-Tc                        | pLAR derivative providing a tetracyclin resistance cassette                | this work  |
| pLAR-Hyg                       | pLAR derivative providing a hygromycin resistance cassette                 | this work  |
| pLAR-Tmp                       | pLAR derivative providing a trimethoprim resistance cassette               | this work  |
| pLAR-Gm                        | pLAR derivative providing a gentamicin resistance cassette                 | this work  |

their biotechnological potential with respect to polysaccharide and vitamin production and Agrobacterium-mediated plant transformation. However, the toolbox of available plasmid vectors is underdeveloped in this group of bacteria.
secondary chromosomes, chromids, or megaplasmids, some of which even exceed 2 Mb in size. For instance, *Mesorhizobium loti* MAFF303099 possesses a chromosome and two megaplasmids (pMla, pMlb), whereas *Sinorhizobium meliloti* Rm1021 harbors a chromosome, a chromid (pSymB), and a megaplasmid (pSymA). *Rhizobium etli* CFN42 even contains six megaplasmids (p42a-f) beside its main chromosome, and *Agrobacterium tumefaciens* C58 maintains two chromosomes, a circular and a linear one, and two plasmids (pTi, pAt). The unrivaled variety of α-rhizobial genome architecture offers a rich resource of megaplasmids that are faithfully inherited to the daughter cells. These are ideal candidates for plasmid vector construction.

DNA replication origins of these extra-chromosomal replicons usually belong to the repC superfamily, named after the DNA replication initiator RepC, which appears to be unique to α-proteobacteria. In repABC family plasmids, repC including the cognate oriV forms an operon with repA and repB. RepA and RepB belong to the ParAB family of partitioning proteins mediating segregation of newly replicated oriVs. Segregation is initiated by specific binding of RepB to its cognate cis-acting centromere-like repS sites. Highly reliable inheritance, even of dispensable repABC-type replicons such as most of the *R. etli* megaplasmids, can be attributed to proper function of the segregation machinery.

Replicon copy number is supposed to be mainly transcriptionally controlled by RepAB-mediated autorepression of the repABC operon and post-transcriptionally by a small counter-transcribed RNA, which is usually encoded by a locus between repB and repC (ctRNA). Strict regulation of repABC expression is the mechanistic basis of incompatibility groups allowing stable coexistence of several repABC replicons in the cell. The unirvaled variety of α-rhizobial genome architecture offers a rich resource of megaplasmids that are faithfully inherited to the daughter cells. These are ideal candidates for plasmid vector construction.

Figure 1. pABC vector concept and assembly strategy. (a) A pABC is composed of up to five module library parts. Modules oriVSm and oriVEC mediate propagation in *S. meliloti* and *E. coli*, respectively. To ensure proper transcriptional control of the repABC operon, the multiple cloning site (yellow box) of the synTer-MCS module, serving as insertion site for the gene load, is flanked by transcriptional terminators (loop structures). A common RK2/RP4 mobilization site (module oriT) enables conjugal transfer of pABCs from *E. coli* to *S. meliloti*. For reliable selection in both organisms standardized antibiotic resistance cassettes were developed (module AR). Each module is flanked by linker sequences for standardized LCR-based assembly (black boxes). In the pABC design, order and orientation of modules is intended to minimize mutual influences of adjacent regions. (b) Detailed scheme of pABC design types A to C. The size of design-derived homologous regions potentially occurring in multi-pABC systems is denoted. Setups C1 and C2 differ in the number of transcription terminator sequences (loop structures) protecting the MCS after Cre-mediated deletion of modules oriVEC and oriT. (c) Module-specific linker sequences (gray and black lines) facilitate LCR-based assembly of module parts with standardized bridging oligonucleotides BO1 to BO4. Integration of the oriT module requires BO3a and BO3b.
group of bacteria. In *S. meliloti*, pABCs were shown to mimic megaplasmid-like characteristics, such as high propagation stability, unit-copy number and combinability of compatible repABC-type plasmids, rendering them highly suitable for complex ectoplastic expression systems. We further devised an in vivo cloning strategy based on Cre/lox-mediated site-specific recombination and an IPTG-inducible engineered oriV_repABC allowing for translocation of DNA fragments to an autonomously replicating repABC-based vector and conjugation-mediated transfer either to compatible rhizobia or to *E. coli*.

**RESULTS AND DISCUSSION**

### Autonomous Plasmid Replication Mediated by Heterologous repABC Regions in *S. meliloti*.

Initially, we tested the ability of various repABC regions to mediate plasmid maintenance in *S. meliloti*, which is a well-characterized model of nitrogen-fixing plant-symbiotic α-rhizobia. To this end, repABC regions derived from megaplasmids of *S. meliloti* (pSymA), *Rhizobium etli* (p42b, p42d, p42e, p42f), *Mesorhizobium loti* (pMla, pMlb), *Sinorhizobium fredii* (pNGR234b), and *Agrobacterium tumefaciens* (pTi) were inserted into the suicide vector pK18mob2 (Table 1), which is replicative in *E. coli*, but nonreplicative in *S. meliloti*.59 Plasmids carrying the repABC cassettes from *R. etli*, *M. loti* and *A. tumefaciens* were successfully established in the *S. meliloti* Rm1021 wild type strain. In contrast, plasmids carrying the repABC regions derived from pSymA, pSMED01, pSMED02, and pNGR234b were not maintained in this strain, probably due to incompatibility between the indigenous megaplasmids and these heterologous replicons derived from closely related α-rhizobia. In line with this observation, the construct harboring the repABCpSymA cassette propagated in the pSymA-cured *S. meliloti* Rm1021 derivative SmA818, but not in the wild type. The identified set of eight heterologous repABC regions autonomously replicating in the *S. meliloti* Rm1021 wild type strain provided the foundation for construction of the family of pABC vectors.

### pABC Vector Design.

Simple design concepts and modularity facilitating assembly and exchange of functional modules have become standard in vector development for applications in synthetic biology.40–43 Figure 1a depicts the blueprint of the pABC vector family composed of five basic modules: oriVSm (repABC cassette for propagation in *S. meliloti*), oriVEc (plasmid-derived origin of replication for propagation in *E. coli*), oriT (RK2/RP4 mobilization site), AR (antibiotic resistance gene cassette), and synTer-MCS (multiple cloning site flanked by transcription terminators).

One of our motivations for developing this vector family is the possibility of coexistence of multiple pABC replicons derived from compatible megaplasmids in one cell. Natural occurrence of α-rhizobial species with up to six compatible repABC-type replicons indicate the high potential of combinability.23 Previously, for a high-throughput plasmid integration study,44 we determined 220 bp sharing at least 80% sequence similarity with the target region as the minimal size promoting homologous recombination at a significant frequency in *S. meliloti* Rm1021. To limit the possibility of homologous recombination between corresponding members of the pABC vector family, parts were designed on the premise of avoiding high and extended sequence identity (Table S1). Multiple parts per module allow for assembly of combinable pABC vectors. The only exception is the oriT module that comprises the ~100 bp common mob site within a 464 bp fragment.99 Because of extended sequence identity, it is therefore not recommendable to combine pABC plasmids with this module in one cell.

Three basic pABC types were devised (Figure 1b). pABCs of type A, lacking the oriT module, have to be introduced to *S. meliloti* by electroporation. pABCs of type B contain an oriT module (pABC-mob) enabling conjugal transfer. Hence, unintended co-integration by homologous recombination between type B pABC vectors through the oriT module appears possible. This is circumvented by design type C which offers the possibility of excision of the oriVEc and oriT modules by Cre/lox-mediated site-specific recombination in *S. meliloti*.45,46

LCR (ligase cycling reaction)17 is our procedure of choice for restriction enzyme-independent assembly of the building blocks

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**Table 2. pABCs Generated in This Study**

| plasmid | synTer-MCS | oriVEc | AR | size (kb) | design type |
|---------|------------|--------|----|-----------|-------------|
| pABCa   | synTer1    | p1SA   | Gm | 6.7       | A           |
| pABCb   | synTer2    | pMB1   | Sp | 6.5       | A           |
| pABCc   | synTer3    | pSC101*| Km | 11.5      | A           |
| pABCc-mob | synTer3  | pSC101*| mob| 12.0      | B           |

$pABCa$, $pABCb$, and $pABCc$ are based on building blocks lacking the module-specific linker sequences and a transcription terminator downstream of the MCS.
to pABC vectors. LCR was shown to efficiently assemble up to two 2 kb DNA fragments. Library plasmids serving as a template for PCR amplification of the 23 parts generated in our study were constructed (Table 1). Each part is flanked by a module-specific linker, which enable LCR-based assembly promoted by the standardized bridging oligonucleotides BO1-4 (Figure 1c, Table S2). Following in vitro assembly, E. coli DH5α was directly transformed with the purified LCR reaction mix, and correct assembly of pABC vectors was verified by DNA sequencing. Usually more than 75% of tested clones exhibited correct assembly, indicating a suitably efficient assembly system.

Library parts for each module (Table 1 and Table S3) and pABC vectors (Table 2 and Table S4) generated in this study are described in the SEVA-SIB database (http://seva.cnb.csic.es). To translate individual pABCs into the SEVA-SIB collection, a unique identifier was assigned to each insert in accordance with an adapted SEVA nomenclature (Tables S3 and S4).

Parts Characterization. oriVSm Module. The common element of the pABC vector family is the repABC region which naturally acts as replication origin of megaplasmids of various α-proteobacteria.20,26 repABC cassettes of different megaplasmids were integrated into pK18mob2 derivative pLoriVSm resulting in library module vectors which provide the repABC cassettes of R. etli p42b,d,e,f (pLoriV-42b, -42d, -42f, -42f1, -42f2, -Mla, -Mlb, and -Ti). Figure 2a depicts the genetic structure of the eight repABC regions, which constitute the basis of the oriVSm module parts used in this study. To test for autonomous propagation and stability in the absence of selective pressure, S. meliloti Rm1021 was transformed with the pLoriVSm library plasmids. Since functional elements, such as partitioning sites (repS, yellow boxes) or the promoter of the antisense RNA incr potentially are located outside of the repABC operon, repABC cassettes were expanded several hundred base pairs up- and downstream of the coding regions. Although a suicide vector carrying the repABC operon of p42d plus 500 bp downstream of the repC stop codon was able to propagate in R. etli CFNX101,77 the downstream region was generously expanded in order to include a further predicted repS site.78 (b) Stability assay of pLoriVSm derivatives. S. meliloti Rm1021 carrying pLoriV-42b, -42d, -42f1, or -Ti, or the shorter constructs p42e_s or pMla_s was grown for 72 h in nonselective TY medium and diluted every 12 h to an OD600 of 0.1 and grown for 12 h in selective TY medium. Error bars indicate the standard deviation calculated from three technical replicates. (c) Growth of S. meliloti Rm1021 carrying selected pLoriVSm plasmids compared to MWSm8 (wild type, Km'). Cultures were adjusted to an OD600 of 0.1 and grown for 12 h in selective TY medium. Error bars indicate the standard deviation calculated from four technical replicates. (d) qPCR-based copy number determination of pLoriVSm derivatives (+/- SD) (see Table S6). SD: standard deviation.

ACS Synthetic Biology Research Article DOI: 10.1021/acssynbio.6b00320 ACS Synth. Biol. 2017, 6, 968−984
plasmids. Restriction enzyme-digested pLoriVSm plasmids purified from the S. meliloti transformants showed the expected electrophoresis banding patterns (Figure S1), verifying autonomous propagation of these replicons in S. meliloti.

pLoriV-42b, -42d, -42e, -Mlb, and -Ti exhibited highly stable propagation despite cultures being kept in exponential growth for 72 h in nonselective rich medium (Figure 2b). The repABC region used for construction of library plasmid pLoriV-42e is characterized by a 542 bp 3’UTR. A shorter variant with a 26 bp 3’UTR (p42e) was less stable, indicating that genetic elements required for faithful replication or segregation of these replicons can be located further downstream of the repABC operon.30 Plasmids harboring oriVp42f, oriVe42f, or oriVp42e derived cassettes were virtually lost in the same period of time. Expanding the 3’UTR of repABC by 45 bp (pMla_s) to 556 bp (pLoriV-Mla) did not improve plasmid maintenance. Slight incompatibility between repABC and the oriV regions of the indigenous megaplasmids may account for this viable but unstable coexistence. Cross-interactions between components of indigenous and heterologous RepABC systems may impair regulatory control of the repABC operons or function of the gene products in replication initiation or plasmid segregation. This may affect cell cycle progression and diminish cell growth. Indeed, pLoriV-42f1, -42f2, and -Mla negatively affected S. meliloti cell growth, whereas stably propagating plasmids bearing oriV cassettes from p42b, p42e, pMla, and pTi had no significant influence on growth (Figures 2c and S2). This is supposed to be a critical prerequisite for a multi-pABC setup under nonselective conditions since a negative effect on cell growth would promote plasmid loss.

We aimed at preserving the unit-copy number or low copy number of megaplasmids in the free-living state as an important attribute of pABC vectors. The copy number of pLoriVSm plasmids in S. meliloti was determined by qPCR and data were normalized to pK18mob2-repABC/SynAwhich in a calibration experiment was shown to exhibit the same copy number as the chromosome (Table S5). The stably propagating pLoriV library plasmids roughly matched a unit-copy number, whereas instable plasmids showed values below 1 (Figure 2d and Table S6). Since a pPCR-based copy number determination was performed on crude cell extracts, a significant number of pLoriV plasmid-negative cells could account for the low values determined for the unstable plasmids pLoriV-42f1 and -42f2 (Figure 2d).

Considering stability and copy number, the oriVSm module vectors pLoriV-42b, -42d, -42e, -Mlb, and -Ti exhibited the desired megaplasmid-like characteristics and thus appear to be highly suitable for construction of the pABC vector system.

**synTer-MCS Module.** This module provides a multiple cloning site offering recognition sites for the rare-cutting restriction enzymes Pmel, Paci, and NotI. Transcriptional read-through into the repABC operon was found to interfere with its strict native expression control. The MCS module was therefore additionally equipped with flanking transcription terminators to protect the oriVSm module and the MCS position from transcriptional read-through emanating from sequences inserted into the MCS or adjacent modules. To this end, synthetic termination regions (synTer1=3) flanking the MCS were designed based on the E. coli rrnBT1 and rrnBT2 terminators (Figure 3a) and cloned into pK18mob2 to generate the pLSynTer library plasmids (Table 1). Effective transcription termination was verified by interrupting expression of an egfp reporter gene from a strong constitutive promoter by inserting the synTer1=3 cassettes downstream of the promoter (Figure S3).

**oriVec and oriT Modules.** The oriVec module enables E. coli-based cloning of pABCs. However, construction of standardized library plasmids carrying a further E. coli replication origin (besides the native oriVpMB1) failed. For this reason, oriVec parts harboring oriVpMB1, oriVe42f, and oriVe42e were PCR-amplified from nonlibrary plasmids by use of extended primers carrying the module specific linker sequences. The oriT module provides an RP4-derived mob site which mediates conjugal transfer from E. coli to a wide range of Gram-negative bacteria including S. meliloti. This module is available on the p LoriT library plasmids (Table 1).
The low variability of oriVEc and oriT module parts is associated with a reduced combinability of pABC vectors since homologous regions provoke unintended replicon fusions. For this reason we equipped the synTer-MCS and oriT modules with Cre recognition (lox) sites, resulting in the pLsynTer-x-lox and pLoriT-x-lox library plasmids, respectively (Figure 3a−b, Table 1). These lox sites allow Cre-mediated deletion of these modules from the pABC vectors in the α-rhizobial host strain SmCreΔhsdR as demonstrated in Figure S4.

**AR Module.** This module comprises selection markers conferring resistance to the antibiotics gentamicin, hygromycin, kanamycin, tetracycline, trimethoprim, and spectinomycin in *E. coli* and *S. meliloti* (Figure S5). For the AR module, we chose a standardized design with the 72 bp Pmin2 promoter driving expression of the resistance genes (Figure 3c). This promoter was selected from PaacC1 and Ptrp derivatives (Figure S6). AR module parts are available on the pLAR library plasmids (Table 1).

**Characterization of pABC Shuttle Vectors.** We constructed 14 pABCs representing design types A to C utilizing all oriVSm module parts described above (Table 2 and Table S7). pABC-a-c are based on constructs lacking the standard linker sequences flanking the module library parts. They basically match the structure of pABC1 to six that are composed of library parts assembled via module-specific linker sequences and include an additional transcription terminator downstream of the MCS (Figure S7a). Dependent on the pABC design, transformation of *S. meliloti* Rm1021 was carried out via electroporation or *E. coli* S17-1 mediated conjugal transfer. Following electroporation, transformation rates of *S. meliloti* Rm1021 with pABCs were massively higher than with the common replicative vectors pSRKGm and pPHU231.45 Compared to pSRKGm, pABCa, pABCb, and pABCa-mob achieved ~7200-, 88-, and 22-fold higher transformation efficiencies, respectively, making cotransformation of *S. meliloti* with up to...
three pABCs possible (Figure S7b). Successful introduction of pABCs into *S. meliloti* and autonomous propagation was verified by plasmid purification from transconjugants and restriction enzyme digest-derived fragment patterns (Figure S8).

Inheritance of pABC vectors was studied by flow cytometry-based single cell analysis applying *S. meliloti* strains carrying pABC-egfp derivatives (Table S8). In contrast to the preliminary selection marker-based assay of maintenance of the pLoriVs library plasmids (Figure 2b), which tends to preselect fast growing cells, the flow cytometry-based assay allows for unbiased determination of the ratio of pABC-positive and-negative cells and is highly sensitive (Figure S9). Cultures were incubated in nonselective rich medium and diluted twice a day to allow for continuous growth in the range of OD<sub>600</sub> of 0.05 to 1.0.

Consistent with the properties of the corresponding pLoriVs library plasmids, derivatives of pABCa (<i>oriV<sub>plsb</sub></i>), pABCb (<i>oriV<sub>plsb</sub></i>), pABC-mob (<i>oriV<sub>pl42a</sub></i>), pABC1 (<i>oriV<sub>pl42a</sub></i>), and pABC4 (<i>oriV<sub>pl42a</sub></i>) were remarkably stably propagated over the time course of 96 h (Figure 4a,i). After 24 h even ~99% of measured cells were eGFP-positive and therefore supposed to maintain the respective pABC replicon. After 96 h of incubation still 97% of cells were positive, indicating a highly reliable propagation of the pABC derivatives in *S. meliloti* Rm1021. In contrast, egfp expressing derivatives of pABC2 (<i>oriV<sub>pl40a</sub></i>) and the low-copy plasmid pHU231 showed a pronounced plasmid loss of ~20% after 24 h (Figure 4a,ii). After 96 h, the proportion of positive cells even decreased to 13% and 39%, respectively. Notably, flow cytometry revealed dramatic variations in propagation stability between biological replicates of clones bearing the <i>oriV<sub>pl77</sub></i>-based pABC3-egfp (99%, 49%, and 2% positive cells after 48 h) (Figure 4a,ii). Mutation of the cognate repABC cassette was excluded by resequencing. However, we cannot exclude mutations in other pABC modules or the *S. meliloti* background genome. Previously, regulatory mechanisms affecting replication and thereby the copy number of pTi in *A. tumefaciens* have been reported.49 The heterogeneity in propagation stability observed in our study and variations in copy number of pTi characterize as very reliably inherited to the daughter cells was less affected by the presence of expR. After 96 h, 63%, 83%, and 92 to 94% of cells contained pABC4, pABCa, and pABCb, respectively. Nevertheless, derivatives of pABCa to pABCc and pABC4 were reliably maintained with 98 to 99% positive cells after 24 h, despite carrying a functional copy of expR throughout the entire time course (Figure S12b). Hence, vectors bearing oriV<sup>+</sup> derivatives from p42b, p42d, p42e, and pMlb exhibit remarkable inheritance even when imposing a fitness burden on the cell.

To validate the unit copy number of stably propagating pABCs and to rule out potential morphological aberrations of pABC-bearing cells, fluorescently labeled pABC derivatives were applied for microscopy analysis. To this end, *Yersinia pestis* (pABC2-egfp) was virtually lost (Figure 4c), whereas the derivative lacking expR (pABC2-egfp) was still present in 17% of the cells (Figure 4a,ii). Maintenance of pABCs characterized as very reliably inherited to the daughter cells was less affected by the presence of expR. After 96 h, 63%, 83%, and 92 to 94% of cells contained pABC4, pABCa, and pABCb, respectively. Nevertheless, derivatives of pABCa to pABCc and pABC4 were reliably maintained with 98 to 99% positive cells after 24 h, despite carrying a functional copy of expR throughout the entire time course (Figure S12b). Hence, vectors bearing oriV<sup>+</sup> derivatives from p42b, p42d, p42e, and pMlb exhibit remarkable inheritance even when imposing a fitness burden on the cell.

In the reference strain Rm1021, expR is disrupted by an insertion element.51 Complementation of this mutation results in a strong increase in galactoglugcan production since the LuxR-type regulator ExpR is a key activator of biosynthesis of these exopolysaccharides.31–35 expR under the control of PluxZ was integrated into pABCs based on repABC cassettes derived from pMlb (pABCa-egfp-expR), p42b (pABCb-egfp-expR), p42d (pABCc-egfp-expR), p42e (pABC4-egfp-expR), and pMla (pABC2-egfp-expR). Constitutive expression of expR significantly decreased cell growth and resulted in a mucoid colony phenotype, indicating a proper function of the expR cassette in the pABCs (Figure S12a). We compared maintenance of expR-positive and expR-negative pABCs reliably and unreliably propagating in *S. meliloti*. After 96 h, the expR-positive derivative of the unreliably propagating pABC2 (pABC2-egfp-expR) was virtually lost (Figure 4c), whereas the derivative lacking expR (pABC2-egfp) was still present in 17% of the cells (Figure 4a,ii). Maintenance of pABCs characterized as very reliably inherited to the daughter cells was less affected by the presence of expR. After 96 h, 63%, 83%, and 92 to 94% of cells contained pABC4, pABCa, and pABCb, respectively. Nevertheless, derivatives of pABCa to pABCc and pABC4 were reliably maintained with 98 to 99% positive cells after 24 h, despite carrying a functional copy of expR throughout the entire time course (Figure S12b). Hence, vectors bearing oriV<sup>+</sup> derivatives from p42b, p42d, p42e, and pMlb exhibit remarkable inheritance even when imposing a fitness burden on the cell.

To validate the unit copy number of stably propagating pABCs and to rule out potential morphological aberrations of pABC-bearing cells, fluorescently labeled pABC derivatives were applied for microscopy analysis. To this end, *Yersinia pestis* and fluorescent repressor operator system (FROS) derived <i>parS</i>/mChr-ParB<sup>−</sup> and <i>teto</i>/TetR-YFP<sup>−</sup> pairs were applied as a taurine-inducible dual color label system. Heterologously expressed DNA-binding proteins mChr-ParB and TetR-YFP bind to <i>parS</i> and <i>teto</i> sequences, respectively, allowing for specific labeling of individual pABCs. Localization patterns of <i>parS</i>/mChr-ParB and <i>teto</i>/TetR-YFP clusters implied single copy replication of pABCa to pABCc, pABC1 and pABC4, since the number of fluorescent foci never exceeded two per cell (Figure S13a). This observation is in accordance with the copy number of pLoriVs library constructs determined by qPCR (Figure 2d). Cell populations of the *S. meliloti* strains with the unreliably inherited pABC2 (oriV<sub>pl60a</sub>), pABC5 (oriV<sub>pl42a</sub>), and pABC6 (oriV<sub>pl42a</sub>) vectors contained a substantial proportion of cells lacking fluorescent foci, indicating loss of the respective vector (Figure S13b). Aberrant cell morphologies, such as branching and elongation, were not observed. Previously, we observed that the repA2B2C2 region derived from pSymA is sufficient to confer the spatiotemporal pattern of this megaplasmid to a small plasmid.58 Although, spatiotemporal dynamics and integration of pABC into the cell cycle must be examined in more detail in future studies, microscopic analyses suggest a megaplasmid-like coordination with subpolar localization in small cells at the beginning of the cell cycle and in elongated predivisional cells (Figure 5a).

A well-defined insulated cloning site is a main requirement for the applicability of the pABC vectors. Insulation was tested by measuring fluorescence derived from promoter-less reporter gene insertions into the cloning site (Figure 5b and S14). Thus, pABCa, pABCb, pABCc, pABC1, and pABC4 are supposed to be the most reliable candidates for applications in *S. meliloti*.50 They are the most reliable candidates for applications in *S. meliloti*.50
an autonomously replicating repABC-type replicon, and demonstrated its feasibility by translocation of the ∼24 kb exo gene cluster from pSymB to a repABC-type replicon (Figure 6).

This strategy requires genomic integration of a repABC-type oriV region adjacent to the region of interest. To enable stable integration, we constructed a LacI-silenced and IPTG-inducible repABC_morph-lacO operon by insertion of a lac operator sequence into the repABC promoter region (Figure 6a). Constitutive lacI expression strongly affected the capacity of pMlb_lacO bearing the engineered repABC_morph-lacO to transform S. meliloti, most probably due to repression of the repABC operon (Figure S15a). Whereas establishment of pMlb_lacO in S. meliloti carrying the medium copy plasmid pSRK-Gm as LacI donor was completely abolished, S. meliloti harboring the single copy plasmid pABCb-lacI exhibited still sufficiently reduced transformation rates. Thus, lacI expression from a single copy locus achieved sufficient inactivation of repABC_morph-lacO.

Figure 6b,i depicts the genomic situation of S. meliloti SmCre_exo-IN bearing plso and Pmin2-loxR-aacC1 integrated up- and downstream of the exo gene cluster, respectively. Whereas the downstream fragment constitutes a functional gentamicin resistance cassette which is separated from the exo gene cluster by the Cre recognition site loxR, plso provides a loxL site which is preceded by a terminator sequence preventing transcription of the promoter-less spectinomycin resistance gene adaA1. plso further offers a constitutively expressed lacI gene encoding the transcriptional repressor which mutates the adjacent repABC_morph_lacI operon in the absence of IPTG. This repression of oriV activity enables integration of plso into the genome. Accordingly, the efficiency of S. meliloti transformation with plso was ∼4000-fold decreased when mating mixtures were incubated on TY agar lacking IPTG and ∼20% of transconjugants carried the construct integrated into the genome (Figure S15a). Induction of Cre expression and simultaneous activation of oriV_morph_lacO by IPTG resulted in excision and maintenance of the floxed DNA fragment, respectively, giving rise to strain S. meliloti SmCre_exo-OUT carrying plso-exo as an autonomous mini-replicon. Successful Cre-mediated recombination, resulting in an active loxP site on plso-exo and loxLR remaining on pSymB, was verified via sequencing (Figure S15b). Since the recombination event caused an antibiotic resistance switch, the screening effort for identifying positive clones was minimized. ABC cloning provided high efficiency rates of ∼75% after Cre/lox-mediated isolation, which can be attributed to highly efficient Cre recombination in SmCre derivatives in general, and suitable function of the engineered repABC-type oriV in particular. Since multiple loci sites recombine in an unpredictable manner, a single copy number of activated repABC_morph-lacO plasmids is an important prerequisite for manageable Cre reactions. Promoter modification appeared to affect the propagation stability of pMlb_lacO and plso-exo, while retaining copy number control, as suggested by the qPCR-based copy number determination (Figure S15c).

Transfer of plso-exo to E. coli was achieved via triparental mating. oriV_morph on the plso backbone enabled replication in E. coli due to the high copy number efficient purification of the large DNA molecule from this host (Figure 6b,iii). Moreover, conjugation-mediated transfer to A. tumefaciens C58 exoB resulted in complementation of the exopolysaccharide-deficient phenotype (Figure 6b,iii), demonstrating propagation of the engineered repABC cassette also in this host (Figure S15d).

Applicability of repABC-Based Plasmids in the Rhizobiales. To further explore the potential applicability of

Figure 5. Examination of pABCs in S. meliloti. (a) Fluorescence microscopy of S. meliloti JDSm106 carrying pABCa-tetO, pABCb, and pABCc-parBYp. Expression of reporters tetR-YFP (yellow) and mCherry-parBYp (red) was induced with 15 mM taurine for 4 h. Snapshots show cells at different stages of the cell cycle. Scale bar: 1 μm. (b) eGFP fluorescence of S. meliloti Rm1021 carrying pABC1-eGFP_1/t (synTer1), pABC1-ST2-eGFP_1/t (synTer2), and pABC1-ST3-eGFP_1/t (synTer3) was taken as a measure for the transcription activity in the MCS of library-derived pABCs. Promoter-less eGFP_1/t cassettes were either forwardly or reversely (corresponding to up/down orientation) integrated into the MCS. Negative: S. meliloti/pABC1; positive: S. meliloti/pABC1-eGFP. Error bars indicate standard deviation calculated from three technical replicates. FU: fluorescence units.
heterologous repABC-based vectors beyond S. meliloti, a pilot survey was performed in Agrobacterium tumefaciens CS8, Mesorhizobium loti MAFF303099, Sinorhizobium medicae CC169, Sinorhizobium fredii NGR234, Rhizobium leguminosarum Norway, Methylobacterium extorquens AM1 Δcel, and Caulobacter crescentus CB15N (Table S9). In the diverse tested members of the Rhizobiaceae, four to seven of the eight probed repABC regions mediated plasmid replication. Even in the distantly related C. crescentus, one of the eight repABC regions (oriV_<sub>repABC</sub>) mediated plasmid replication, although growth on medium selecting for presence of this repABC-based plasmid was impaired. This may indicate unreliable replication or segregation of this plasmid, or interference with cellular functions in C. crescentus. The initial survey of the pilot set of repABC modules in α-proteobacteria suggests that this vector family is still cumbersome and integration sites may in number problems, stable integration of multiple constructs is number ranges and cell-to-cell variation complicate balanced gene expression even from a set of compatible plasmids. While chromosomal integration of expression constructs solves copy number problems, stable integration of multiple constructs is still cumbersome and integration sites may influence gene expression. In this study, we established the pABC vector family which circumvents these issues and highly facilitates the setup of combinatorial expression systems.

pABC vectors provide a well-defined integration site in single copy and we demonstrated stable coexistence of up to three compatible pABCs. Occurrence of α-rhizobial species harboring up to six RepABC-family plasmids suggests that even more than three pABC plasmids may be combined in a suitable host. The number of available resistance cassettes and restraints in the applicability of multiple antibiotics selection pressure potentially hampers combinatorial use of pABC vectors. However, oriV<sub>repABC</sub>-based multireplicon systems benefit from robust inheritance under nonselective conditions. A future route to further stabilization of pABC plasmids, independent of antibiotic...
resistance cassettes, could be the integration of toxin–antitoxin (TA)-based maintenance systems, which frequently occur in *S. meliloti*. Integration of additional modules is facilitated by the modular PABC design, which also facilitates construction of pABCs with multifarious part combinations. pABC plasmids were designed as shuttle vectors capable of replication in *E. coli* and *S. meliloti*. This is an advantageous feature when it comes to *E. coli*-based cloning procedures and plasmid DNA purification. Nevertheless, some applications in *S. meliloti* may favor pABC plasmids lacking oriVEc and oriT. We demonstrated that these modules can be efficiently removed on demand by induction of Cre/lox-mediated site specific recombination in *S. meliloti*.

Furthermore, we took a stab at the broad potential of pABC applications by devising the *in vivo* ABC cloning procedure, which provides high flexibility due to isolation and immediate maintenance of the fragment of interest in a repABC-based vector. This opens up multifarious follow-up processes including intracellular relocation of the isolated fragment by Cre/lox mediated recombination or conjugal transfer to a compatible host organism. In a future perspective, the available set of non-promiscuous lox spacer mutants paves the way to serial engineering steps, such as *in vivo* assembly of indigenous or heterologous DNA fragments on pABC plasmids. Thus, the family of PABC vectors has the potential to significantly advance metabolic and genome engineering in *S. meliloti* and related species and to provide a platform for *in vivo* DNA assembly.

**METHODS**

**Bacterial Strains and Growth Conditions.** Bacterial strains used in this study are derivatives of *Escherichia coli* K12, *Sinorhizobium meliloti* Rm1021, *Agrobacterium tumefaciens* C58, *Rhizobium etli* CFN42, *Sinorhizobium fredii* NGR234, *Mesorhizobium loti* MAFF303099, *Rhizobium leguminosarum* Norway, *Methylobacterium extorquens* AM1 Δcel, *Sinorhizobium medicae* WSM419 and CC169, and *Caulobacter crescentus* CB15N. *E. coli* strains were cultivated at 37 °C, whereas *A. tumefaciens*, *M. loti*, *R. etli*, *S. fredii*, and *S. medicae* were grown at 30 °C in Luria–Bertani (LB) medium.68 *S. meliloti* strains were cultivated at 30 °C in tryptone yeast extract (TY) medium.69 *C. crescentus* was grown at 28 °C in tryptone-yeast extract (PYE) medium.70 If necessary, the following antibiotics were used: gentamicin (8 μg/mL for *E. coli*, 30 μg/mL for *S. meliloti*, and 50 μg/mL for *A. tumefaciens*), kanamycin (25 μg/mL for *C. crescentus*, 30 μg/mL for *S. fredii*, and *R. leguminosarum*, 50 μg/mL for *E. coli*, *M. loti*, and *M. extorquens*, 70 μg/mL for *A. tumefaciens*, 200 μg/mL for *S. meliloti*), streptomycin (600 μg/mL for *S. meliloti* and *R. leguminosarum*), spectinomycin (100 μg/mL for *E. coli*, 200 μg/mL for *S. meliloti*, and 500 μg/mL for *A. tumefaciens*), hygromycin (100 μg/mL for both *E. coli* and *S. meliloti*), naldixic acid (10 μg/mL for *A. tumefaciens*, *S. fredii*, *M. loti*, and *S. medicae*), trimethoprim (1000 μg/mL for *E. coli*, 700 μg/mL for *S. meliloti*), chloramphenicol (20 μg/mL for *E. coli*) or tetracycline (10 μg/mL for *E. coli* and 3 μg/mL for *S. meliloti*). Cre/lox applications were performed as described before,46 excluding tetracycline supplementation during induction of cre expression. Calcofluor fluorescence assays of *A. tumefaciens* CS8 exoB carrying pLS2-exoB were performed on LB agar supplemented with gentamycin and spectinomycin, 200 μg/mL calcofluor, and 500 μM IPTG. Cultures of an optical density at 600 nm (OD600) of 0.2 were spotted on agar medium and grown overnight at 30 °C. Calcofluor fluorescence was excited by UV transillumination.

**DNA Manipulation and Plasmid Extraction.** Standard techniques were employed for common cloning procedures.58 Primers and PCR products were 5′-phosphorylated applying T4 Polynucleotide Kinase (Thermo Scientific, Germany). Dephosphorylation of 5′-ends of DNA was achieved by use of FastAP Thermosensitive Alkaline Phosphatase (Fermentas). Blunting of DNA ends was performed by use of DNA Polymerase I (Large Klenow fragment) (New England Biolabs (U.K.)). Plasmid DNA was generally purified using the “E.Z.N.A. Plasmid Mini Kit” (Omega Bio-Tek). For gel extractions of DNA the “illustra GFX PCR DNA and Gel Band Purification Kit” (GE Healthcare Life Sciences) was employed.

**Plasmid and Strain Construction.** Strains and constructs generated in this study are listed in Table 1 and Table S10. Assembled pABCs are listed in Table 2. A detailed description of plasmids is given in Tables S7 and S11. Oligonucleotides and synthetic DNA fragments were provided by Sigma-Aldrich (USA) and Integrated DNA Technologies (USA), respectively (Table S2). DNA fragments were PCR amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs) or Taq DNA Polymerase (New England Biolabs). DNA sequence verification was performed by Eurofins Genomics (Germany). For transformation of *S. meliloti*, the respective strains were either conjugated or electroporated as described before.15 Preparation of electrocompetent cells was done as described by Ferré et al. (2010).

Construction of pABC mini-replicons: Amplification of module parts from library plasmids was performed with Q5 polymerase applying module specific primer sets oriVSm_for/rev (pLoriVSm derivatives), synTer-MCS_for/rev (pLSynTer derivatives), oriT_for/rev (pLoriT derivatives), and AR_for/rev (pLAR derivatives). oriVEc module parts were amplified from pK18mob2 (pMB1), pACYC184 (p15A), and pX-G10 (pSC101*) with primer sets pMB1_for/rev, p15A_for/rev, and pSC101*_for/rev, respectively. Subsequently, matching DNA fragments (blunt-end and 5′ phosphorylated) were fused via ligation cycling reaction (LCR) according to de Kok et al. (2014). For LCR 0.3 U Taq ligase (New England Biolabs), 3 nM DNA parts, 30 nM bridging oligos, and 8% (v/v) DMSO were applied. The following conditions were used: 2 min at 94 °C and then 50 cycles of 10 s at 94 °C, 30 s at 60 °C, and 60 s at 65 °C, followed by incubation at 4 °C. Reaction mixtures were purified using the E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek). A detailed pABC construction manual is provided in the Supporting Information.

For live-cell localization studies of pABC vectors, a dual label system based on the fluorescent reporter gene fusions tetR–YFP (derived from the fluorescent repressor operator system, FROS57) and mChr-parByp (parB derived from Yersinia pestis) was developed. Homologous regions comprising the upstream region of *tuaA* (pSymB position 1050453–1050952) and *PtauA–tauA* (pSymB position 1050781–1051458) were integrated into pK18mob2C, thereby flanking tetR–YFP-mCh-parByp. *S. meliloti* Rm1021 was transformed with the resulting plasmid pJD120. Loss of the plasmid backbone was forced by sucrose selection resulting in strain JDSm106 carrying the tetR–YFP-mCh-parByp reporter construct stably integrated into the chromosome downstream of the *tuaA* promoter. Localization studies of pABCs carrying either a pLAU44-derived tetO array (position 5117-9290) or a pMT1-derived, 86 bp parSy sequence77 were performed in this dual label *S. meliloti* reporter strain JDSm106.
Control strains for cell growth and plasmid inheritance experiments were generated as follows. A homologous region comprising exsA (pSymB position 1166189-1166773) was cloned into pk18mob2, resulting in pMW1. Integration of a P5min2-egfp cassette upstream of the exsA region in pMW1 gave rise to pMW28. pMW1 was integrated into pSymB in S. meliloti Rm1021 by homologous recombination, resulting in the kanamycin resistant strain MWSm8. Integration of pMW28 into the genome of S. meliloti ΔhsdR by homologous recombination gave rise to strain MWSm37 exhibiting constitutive egfp expression.

An S. meliloti strain suitable for ABC cloning of the exo gene cluster was constructed in a two-step cloning strategy applying cloning-free genome editing (CFGE).45 P5min2-loxR-aacC1 (PCR amplified from pJD211 with primers Uni/Rev) was blunt-end ligated with a ~550 bp fragment covering the exoP-thi intergenic region (pSymB position 1190665-1191220; PCR amplified with primers 86/87). Purified ligation products were then introduced to SmCreΔhsdR by electroporation. Finally, a transformant exhibiting P5min2-loxR-aacC1 correctly integrated into the genome downstream of the exo gene cluster was transformed with pSlso, giving rise to strain SmCreexo-IN.

DNA Transfer. Preparation of electrocompetent cells and transformation of S. meliloti, A. tumefaciens, R. etli, S. fredii, M. loti, R. leguminosarum, and S. medicae was performed as described by Ferri et al., 2010. Electroporation of M. extorquens and C. crescentus was carried out as described previously.72,73

To transfer plSO-exo from S. meliloti into different species, triparental matings between S. meliloti SmCreexo-OUT and E. coli XL1blue-pRK2013, with A. tumefaciens C58 exoB or E. coli AB3219 were performed.

For triparental matings the cell density of fresh overnight cultures was adjusted to 2.0 and appropriate dilutions were plated on selective LB agar containing 500 μg/mL chloramphenicol. Integration of pISO-exo from S. meliloti in 1 mL sterile 0.9% (w/v) NaCl solution. For the determination of conjugation efficiency was determined by plating the conjugation efficiency, OD600 of the cell suspension was divided by OD600 of the cell recipient and helper strain were mixed. A 50 μL aliquot of cell suspension was dropped and incubated overnight. Cells were recovered in 1 mL sterile 0.9% (w/v) NaCl solution. For the determination of conjugation efficiency, OD600 of the cell suspension was adjusted to 2.0 and appropriate dilutions were plated on selective LB agar containing 500 μM IPTG. The efficiency of conjugation was determined by plating the conjugation cell suspension on media selective for transconjugants as well as on media selective for all recipient cells. Conjugation frequency was expressed as the number of colony forming units (CFU) transconjugants per CPU recipient cells.

Plasmid Stability Assays. To assess inheritance stability of pLoriVSm derivatives, S. meliloti cultures were grown over 72 h at 30 °C in TY medium supplemented with streptomycin. Cell suspensions were diluted every 12 h to an OD600 of 0.05. Finally, dilution series were plated on nonselective TY agar and incubated at 30 °C for 15 min served as template. The DNA concentration of cell lysates containing pLoriVSm derivatives was verified via Qubit fluorometric quantitation (HS Assay Kit, Thermo Fisher Scientific). qPCR was carried out in a qTOWER Thermal Cycler (Analytik Jena, Germany) using the TaqyonNo Rox SYBRMasterMix dTTTP Blue Kit. Reactions were performed according to the manufacturer’s instructions in a 5 μL reaction volume. Replicon copy number was calculated according to Lee et al. (2006).74

Microscopy. Phase contrast and fluorescence pictures of S. meliloti were taken using an Eclipse Ti-E inverse research microscope (Nikon) and the live imaging modus of the NIS elements software (Nikon). For fluorescence microscopy with a 100x CFI Apo TIRF Oil objective (numerical aperture of 1.49) a green DPSS solid state laser (561 nm, 50 mW; Sapphire) and a multilane Argon laser (65 mW; Melles Griot) were applied with AHP HC filter sets F36-504 TxRed (excitation bp 562/40 nm, beam splitter 593 nm, emission bp 624/40 nm) and F36-513 YFP (excitation bp 500/24 nm, beam splitter 520 nm, emission bp 542/27 nm). Background fluorescence was reduced by applying the highly inclined laminated optical sheet (HILO) technique.75 Cell cultures were grown in TY medium supplemented with suitable antibiotics to an OD600 of 0.1 and expression of the fluorescent labeling system was induced with 15 mM taurine for 4 h. Living cells were placed onto 1% (w/v) TY agarose pads.

Bioinformatics Tools. To generate neutral orthogonal DNA sequences for module linker fragments, the web-based tool R2oDNA Designer was applied. To estimate the recombination potential of distinct pABCs we investigated the local sequence homology of all module parts (including linker sequences). We applied a window-based local alignment approach to focus on the local sequence homology. For a pair of parts we therefore extracted all 200 bp sequence fragments of one part and performed a local alignment on the full sequence of the second part. The best alignment similarity of all 200 bp fragments with the second vector was taken as the similarity value of both vectors.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00320.

Table S1: Examination of sequence identity of pABC module parts. Table S2: Primers and synthetic DNA fragments used in this study. Table S3: Designation of pABC library parts according to the SEVA-SIB nomenclature. Table S4: Translation of pABC plasmids into the SEVA-SIB collection. Table S5: qPCR indicates a unit copy number of plasmids carrying a repABC_SymA cassette. Table S6: Copy number determination of oriVSm library plasmids. Table S7: Detailed description of pABCs generated in this study. Table S8: Flow cytometry measurements of S. meliloti Rm1021 carrying pABC-egfp derivatives. Table S9: Replication capacity of repABC-based replication origins in various α-proteobacteria. Table S10: Strains and plasmids used in Supporting Information. Table S11: Detailed description of constructs
generated in this study. Figure S1. Electrophoresis banding patterns of oriVSm library plasmids. Figure S2. Growth of *S. meliloti* carrying stably propagating pLoriVSm library plasmids. Figure S3. Activity of *E. coli* transcription terminators derived from *rrnBT1* and *rrnBT2* in *S. meliloti*. Figure S4. Cre-mediated deletion of modules oriVEc and oriT in *S. meliloti* strain SmCreΔhsdR. Figure S5. Examination of standardized antibiotic resistance cassettes. Figure S6. Examination of promoter activity of *P* *trp* derivatives in *S. meliloti*. Figure S7. pABC setup and electroporation efficiency. Figure S8. Electrophoresis banding pattern of digested pABCs purified from *S. meliloti*. Figure S9. Verification of Flow Cytometry measurements. Figure S10. Propagation stability of pABC3-egfp and pABC4-egfp. Figure S11. Growth of *S. meliloti* Rm1021 harboring stably propagating pABCs. Figure S12. Characterization of pABC-egfp-expr derivatives. Figure S13. Fluorescence microscopy analysis of fluorescently labeled pABCs. Figure S14. Analysis of transcriptional insulation of the multiple cloning site of pABCα-c. Figure S15. Examination of ABC cloning procedures (PDF)

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J.D. and M.W. contributed equally. A.B., J.D., and M.W. designed the research. J.D., M.W., and C.H. performed the experimental work. M.C., T.J.E., and M.T. contributed to the designed the research. J.D., M.W., and C.H. performed the and M.W. analyzed all data. J.D. and A.B. wrote the paper.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Gabriele Malengo for supporting the FACS analyses, Daniela Kiekebusch and Maritha Lippmann for technical assistance in *Caulobacter* handling, and Macarena Marin for providing *R. leguminosarum* strain Norway. Financial support from the German Research Foundation (Collaborative Research Centre TTR 174 “Spatiotemporal dynamics of bacterial cells” and INST 160/S36-1 FUGG), the LOEWE program of the State of Hesse (Germany) (SYNMIKRO), the Max Planck Society, and the European Research Council (ERC 637675 “SYBORG”) is acknowledged.

**ABBREVIATIONS**

Clm, chloramphenicol; Gm, gentamicin; h, hour; Hyg, hygromycin; Km, kanamycin; oriV, replication origin; SD, Shine–Dalgarno sequence; Sp, spectinomycin; Str, streptomycin; Ta, toxin–antitoxin; Tc, tetracycline; Tmp, trimethoprim

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Research Article

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