

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

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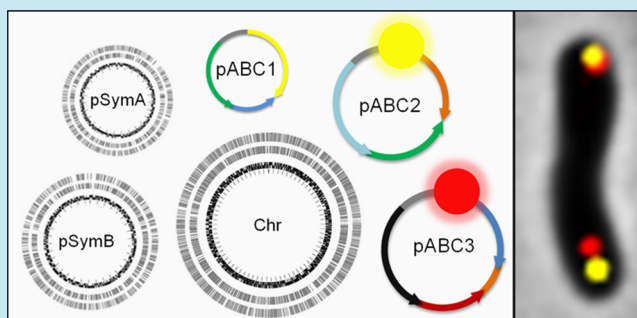
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Supporting Information

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 elemental 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.¹ Plasmids can be considered as ideal DNA-carrying vectors with respect to the ease of plasmid engineering, modularization, and standardization.² 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,^{3–5} and also engineered or synthetic chromosomes potentially offer high propagation stability and cloning capacity.^{6–8}

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^{9,10} or as plant pathogens of the genus *Agrobacterium* causing crown gall tumors on many vascular plants.^{11,12} 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,¹³ but also

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Table 1. Bacterial Strains and Plasmids Used in This Study

strains/plasmids	description	refs
<i>S. meliloti</i>		
Rm1021	wild type strain	22
Δ hsdR	Rm1021 strain with <i>hsdR</i> deletion	45
SmCre Δ hsdR	Rm1021 <i>cre</i> expression strain with <i>hsdR</i> deletion, <i>tauX:: cre-tetRA</i> (Tc ^r)	45
SmA818	strain 2011 cured of megaplasmid pSymA	79
JDsm106	Rm1021 bearing <i>tetR-YFP-mChr-parB_{Yp}</i> (pJD120) under control of <i>PtauAB</i>	this study
MWsm8	Rm1021 bearing pMW1 genomically integrated (Km ^r)	this study
MWsm37	Δ hsdR bearing pMW28 for constitutive <i>egfp</i> expression (Km ^r)	this study
SmCre _{exo-IN}	SmCre Δ hsdR bearing pIso and <i>Pmin2-loxR-aacC1</i> integrated up- and downstream of the <i>exo</i> gene cluster, respectively (Km ^r , Gm ^r)	this study
SmCre _{exo-OUT}	SmCre Δ hsdR carrying pIso-exo isolated from pSymB (IPTG dependent) (Km ^r , Spec ^r)	this study
<i>A. tumefaciens</i>		
C58	wild type strain	24, 25
C58 <i>exoB</i>	exhibiting a CFGE-mediated inactivation of <i>exoB</i> (Gm ^r)	45
<i>exoB</i> /pIso-exo	C58 <i>exoB</i> carrying pIso-exo (IPTG dependent) (Gm ^r , Km ^r , Spec ^r)	this study
<i>R. etli</i>		
CFN42	wild type strain	80
<i>S. medicae</i>		
WSM419	wild type strain	81
CC169	wild type strain	82
<i>S. fredii</i>		
NGR234	wild type strain	83
<i>M. loti</i>		
MAFF303099	wild type strain	21
<i>R. leguminosarum</i>		
Norway	wild type strain	84
<i>M. extorquens</i>		
AM1 Δ cel	strain AM1 deficient in cellulose production	85
<i>C. crescentus</i>		
CB15 N	wild type strain	86
<i>E. coli</i>		
DH5 α	used for cloning procedures and plasmid extraction	87
S17-1	used for conjugation with <i>S. meliloti</i>	88
XL1-Blue	used as recipient of pRK2013 (Tc ^r)	Stratagene
XL1Blue-pRK2013	used for triparental matings (Tc ^r , Km ^r)	this study
AB3219	used as Clm resistant recipient of pISO-exo (Clm ^r)	89
Plasmids		
pK/G18mob2	suicide vector carrying replication origin pMB1	39
pK18mobsacB	suicide vector carrying <i>sacB</i> 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 <i>E. coli</i>)	91
pACYC184	carrying replication origin p15A	92
pPHU231	broad-host-range expression vector	93
pRK2013	helper plasmid for mobilization of nonself-transmissible plasmids	94
pLAU44	pUC18 derivative carrying <i>tet</i> operator (<i>tetO</i>) cassettes	57
pART	artificial mini-replicon based on a pSymA derived <i>repA2B2C2</i> cassette	58
pK18mob2-repABC _{pSymA}	pK18mob2 carrying a pSymA derived <i>repA2B2C2</i> cassette	this work
p42b_p	pK18mob2 carrying <i>repABC</i> _{p42b} based <i>oriV</i> (<i>R. etli</i>)	this work
p42d_p	pK18mob2 carrying <i>repABC</i> _{p42d} based <i>oriV</i> (<i>R. etli</i>)	this work
p42e_s	pK18mob2 carrying a truncated <i>repABC</i> region derived from p42e (<i>R. etli</i>)	this work
p42f1_p	pK18mob2 carrying <i>repABC</i> _{p42f1} based <i>oriV</i> (<i>R. etli</i>)	this work
p42f2_p	pK18mob2 carrying <i>repABC</i> _{p42f2} based <i>oriV</i> (<i>R. etli</i>)	this work
pMla_s	pK18mob2 carrying a truncated <i>repABC</i> region derived from pMla (<i>M. loti</i>)	this work
pMlb_p	pK18mob2 carrying <i>repABC</i> _{pMlb} based <i>oriV</i> (<i>M. loti</i>)	this work
pSMED01_p	pK18mob2 carrying <i>repABC</i> _{pSMED01} based <i>oriV</i> (<i>S. medicae</i>)	this work
pSMED02_p	pK18mob2 carrying <i>repABC</i> _{pSMED02} based <i>oriV</i> (<i>S. medicae</i>)	this work
pNGR234b_p	pK18mob2 carrying <i>repABC</i> _{pNGR234b} based <i>oriV</i> (<i>S. fredii</i>)	this work
pTi_p	pK18mob2 carrying <i>repABC</i> _{pTi} based <i>oriV</i> (<i>A. tumefaciens</i>).	this work
pMW1	integrative pK18mob2 derivative conferring Km ^r	this work
pMW28	integrative pK18mob2 derivative carrying <i>Pmin2-egfp</i>	this work
pJD120	pK18mobsacB derivative, integration of <i>tetR-YFP-mChr-parB_{Yp}</i> under control of <i>PtauAB</i>	this work

Table 1. continued

strains/plasmids	description	refs
Plasmids		
pJD211	pK18mob2 derivative carrying <i>Pmin2-loxR-aacC1</i>	this work
pPHU231-egfp	pPHU231 derivative carrying a constitutive <i>egfp</i> cassette	this work
pABCa-egfp	pABCa carrying a constitutive <i>egfp</i> cassette	this work
pABCa-egfp-expR	pABCa carrying constitutively expressed <i>egfp-expR</i>	this work
pABCa-tetO	pABCa carrying a <i>tetO</i> array	this work
pABCb-egfp	pABCb carrying a constitutive <i>egfp</i> cassette	this work
pABCb-egfp-expR	pABCb carrying constitutively expressed <i>egfp-expR</i>	this work
pABCc-egfp-expR	pABCc carrying constitutively expressed <i>egfp-expR</i>	this work
pABCc-par _{S_{Yp}}	pABCc carrying <i>parS_{Yp}</i>	this work
pABCc-mob-egfp	pABCc-mob carrying a constitutive <i>egfp</i> cassette	this work
pABC1-egfp	pABC1 carrying a constitutive <i>egfp</i> cassette	this work
pABC1-egfp-expR	pABC1 carrying constitutively expressed <i>egfp-expR</i>	this work
pABC1-egfp_f	pABC1 carrying a forwardly integrated SD- <i>egfp</i> fragment	this work
pABC1-egfp_r	pABC1 carrying a reversely integrated SD- <i>egfp</i> fragment	this work
pABC1-T2-egfp_f	pABC1-T2 carrying a forwardly integrated SD- <i>egfp</i> fragment	this work
pABC1-T2-egfp_r	pABC1-T2 carrying a reversely integrated SD- <i>egfp</i> fragment	this work
pABC1-T3-egfp_f	pABC1-T3 carrying a forwardly integrated SD- <i>egfp</i> fragment	this work
pABC1-T3-egfp_r	pABC1-T3 carrying a reversely integrated SD- <i>egfp</i> fragment	this work
pABC2-egfp	pABC2 carrying a constitutive <i>egfp</i> cassette	this work
pABC2-egfp-expR	pABC2 carrying constitutively expressed <i>egfp-expR</i>	this work
pABC3-egfp	pABC3 carrying a constitutive <i>egfp</i> cassette	this work
pABC4-egfp	pABC4 carrying a constitutive <i>egfp</i> cassette	this work
pABC4-egfp-expR	pABC4 carrying constitutively expressed <i>egfp-expR</i>	this work
pABCb-lacI	pABCb carrying <i>lacI</i> for constitutive LacI expression	this work
pMlb_lacO	pK18mob2 carrying <i>repABC_{pMlb}</i> bearing a <i>lacO</i> cassette at the predicted transcription start site	this work
pIso	ABC cloning vector integrating upstream of the <i>exo</i> gene cluster on pSymB	this work
pIso-exo	isolated ABC cloning vector carrying the <i>exo</i> gene cluster (replicative upon IPTG supplementation)	this work
pABC Library Plasmids Carrying Module Parts		
pLoriVSm	pK18mob2 derivative, unloaded library vector for oriVSm parts	this work
pLsynTer-MCS	pK18mob2 derivative, unloaded library vector for synTer-MCS parts	this work
pLoriT	pK18mob2 derivative, unloaded library vector for oriT parts	this work
pLAR	pK18mob2 derivative, unloaded library vector for AR parts	this work
pLoriV-42b	pLoriVSm derivative providing the <i>oriV_{p42b}</i> region (<i>R. etli</i>)	this work
pLoriV-42d	pLoriVSm derivative providing the <i>oriV_{p42d}</i> region (<i>R. etli</i>)	this work
pLoriV-42e	pLoriVSm derivative providing the <i>oriV_{p42e}</i> region (<i>R. etli</i>)	this work
pLoriV-42f1	pLoriVSm derivative providing the <i>repA1B1C1</i> based <i>oriV_{p42f}</i> region (<i>R. etli</i>)	this work
pLoriV-42f2	pLoriVSm derivative providing the <i>repA2B2C2</i> based <i>oriV_{p42f}</i> region (<i>R. etli</i>)	this work
pLoriV-Mla	pLoriVSm derivative providing the <i>oriV_{pMla}</i> region (<i>M. loti</i>)	this work
pLoriV-Mlb	pLoriVSm derivative providing the <i>oriV_{pMlb}</i> region (<i>M. loti</i>)	this work
pLoriV-Ti	pLoriVSm derivative providing the <i>oriV_{pTi}</i> region (<i>A. tumefaciens</i>)	this work
pLsynTer-1	pLsynTer-MCS derivative providing <i>synTer1</i> -MCS	this work
pLsynTer-2	pLsynTer-MCS derivative providing <i>synTer2</i> -MCS	this work
pLsynTer-3	pLsynTer-MCS derivative providing <i>synTer3</i> -MCS	this work
pLsynTer-1-lox	pLsynTer-MCS derivative providing <i>synTer1</i> -MCS- <i>loxL</i>	this work
pLsynTer-2-lox	pLsynTer-MCS derivative providing <i>synTer2</i> -MCS- <i>loxL</i>	this work
pLsynTer-3-lox	pLsynTer-MCS derivative providing <i>synTer3</i> -MCS- <i>loxL</i>	this work
pLoriT-1	pLoriT derivative providing a <i>mob</i> site	this work
pLoriT-1-lox	pLoriT derivative providing <i>mob-loxR</i>	this work
pLoriT-1-loxT	pLoriT derivative providing <i>mob-loxR-rmBT2</i>	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^{14,15} and vitamin^{16,17} production and *Agrobacterium*-mediated plant transformation.¹⁸ However, the toolbox of available plasmid vectors is underdeveloped in this group of bacteria.

In α -proteobacteria, genome segmentation is a common phenomenon. Especially the fast-growing rhizobia harbor multipartite genomes which are usually composed of a main chromosome and additional low or unit-copy replicons classified as

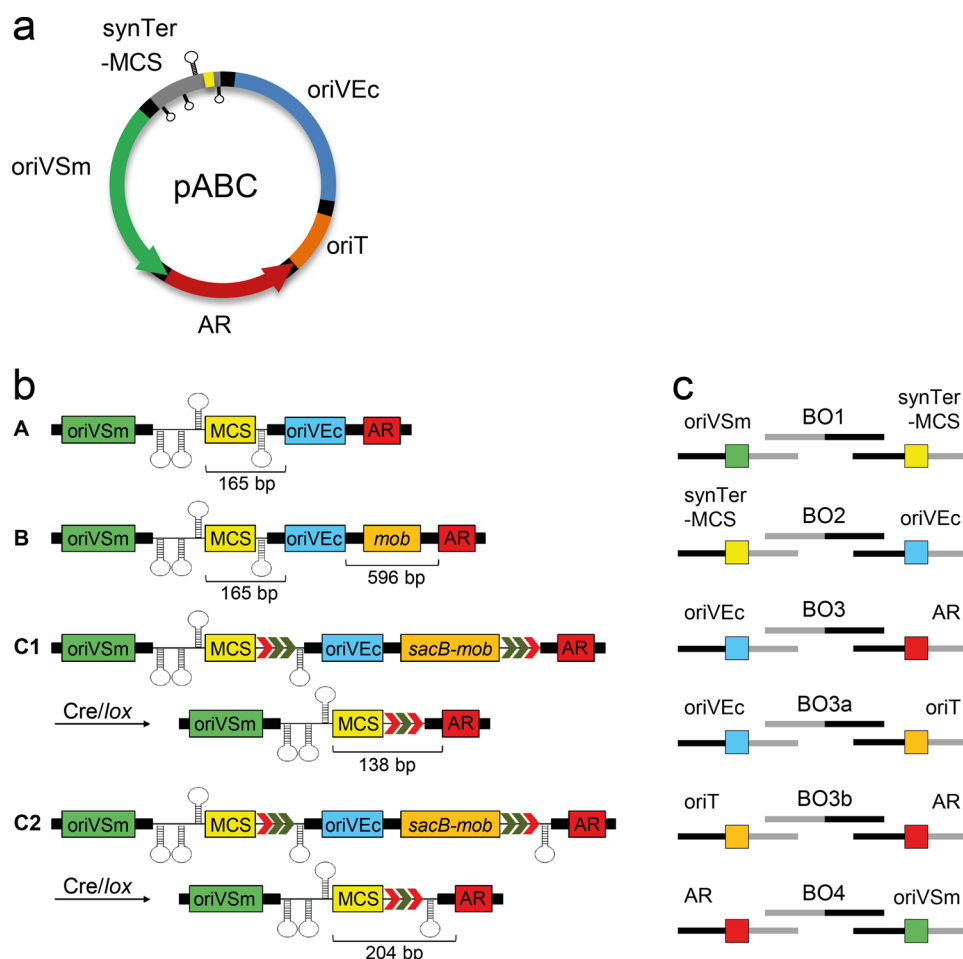


Figure 1. pABC vector concept and assembly strategy. (a) A pABC is composed of up to five module library parts. Modules oriVSm and oriVEc mediate propagation in *S. meliloti* and *E. coli*, respectively. To ensure proper transcriptional control of the *repABC* operon, the multiple cloning site (yellow 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.

secondary chromosomes, chromids, or megaplasmids,^{19,20} some of which even exceed 2 Mb in size. For instance, *Mesorhizobium loti* MAFF303099 possesses a chromosome and two megaplasmids (pMla, pMlb),²¹ whereas *Sinorhizobium meliloti* Rm1021 harbors a chromosome, a chromid (pSymB), and a megaplasmid (pSymA).²² *Rhizobium etli* CFN42 even contains six megaplasmids (p42a-f) beside its main chromosome,²³ and *Agrobacterium tumefaciens* C58 maintains two chromosomes, a circular and a linear one, and two plasmids (pTi, pAt).^{24,25} 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,^{20,26,27} 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*.²⁷ RepA and RepB belong to the ParAB family of partitioning proteins²⁷ mediating segregation of newly replicated *oriVs*. Segregation is

initiated by specific binding of RepB to its cognate *cis*-acting centromere-like *repS* sites.²⁷ Highly reliable inheritance, even of dispensable *repABC*-type replicons such as most of the *R. etli* megaplasmids,^{28–30} can be attributed to proper function of the segregation machinery.^{31,27,32}

Replicon copy number is supposed to be mainly transcriptionally controlled by RepAB-mediated autorepression of the *repABC* operon^{20,26} and post-transcriptionally by a small counter-transcribed RNA, which is usually encoded by a locus between *repB* and *repC* (ctRNA).^{26,33,34} Strict regulation of *repABC* expression is the mechanistic basis of incompatibility groups allowing stable coexistence of several *repABC* replicons in the cell.^{20,27,34–37} High variability of incompatibility factors, such as *repS* sites, the ctRNA and occasionally the C-terminal domain of RepC, may explain the compatibility of heterologous megaplasmids in related species.³⁸

Here, we explored the naturally occurring low or unit-copy number extrachromosomal elements of members of the *Rhizobiaceae* and *Phyllobacteriaceae* to design the versatile family of modular standardized pABC plasmids for this important

Table 2. pABCs Generated in This Study^a

plasmid	modules					size (kb)	design type
	oriVSm (<i>oriV</i> origin)	synTer-MCS (relevant fragment)	oriVEc (<i>oriV</i> origin)	oriT (relevant fragment)	AR (resistance)		
No Linker							
pABCa	pMlb	<i>synTer1</i>	p15A		Gm	6.7	A
pABCb	p42b	<i>synTer2</i>	pMB1		Sp	6.5	A
pABCc	p42d	<i>synTer3</i>	pSC101*		Km	11.5	A
pABCc-mob	p42d	<i>synTer3</i>	pSC101*	<i>mob</i>	Km	12.0	B
Module Library Based Construction							
pABC1	p42b	<i>synTer1</i>	pMB1		Sp	6.7	A
pABC1-loxA	p42b	<i>synTer1-loxL</i>	pMB1	<i>mob-loxR</i>	Sp	9.5	C1
pABC1-loxB	p42b	<i>synTer1-loxL</i>	pMB1	<i>mob-loxR-rnnBT2</i>	Sp	9.6	C2
pABC1-sT2	p42b	<i>synTer2</i>	pMB1		Sp	6.7	A
pABC1-sT3	p42b	<i>synTer3</i>	pMB1		Sp	6.7	A
pABC2	pMla	<i>synTer2</i>	p15A		Gm	7.2	A
pABC3	pTi	<i>synTer3</i>	pSC101*		Hyg	8.6	A
pABC4	p42e	<i>synTer1</i>	pMB1		Sp	7.0	A
pABC5	p42f1	<i>synTer2</i>	pMB1		Sp	7.2	A
pABC6	p42f2	<i>synTer2</i>	pMB1		Sp	7.3	A

^apABCa, pABCb, and pABCc are based on building blocks lacking the module-specific linker sequences and a transcription terminator downstream of the MCS.

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 ectopic 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 medicae* (pSMED01, pSMED02), *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*.³⁹ 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 *repABC_{pSymA}* 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.³⁹ 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)⁴⁷ is our procedure of choice for restriction enzyme-independent assembly of the building blocks

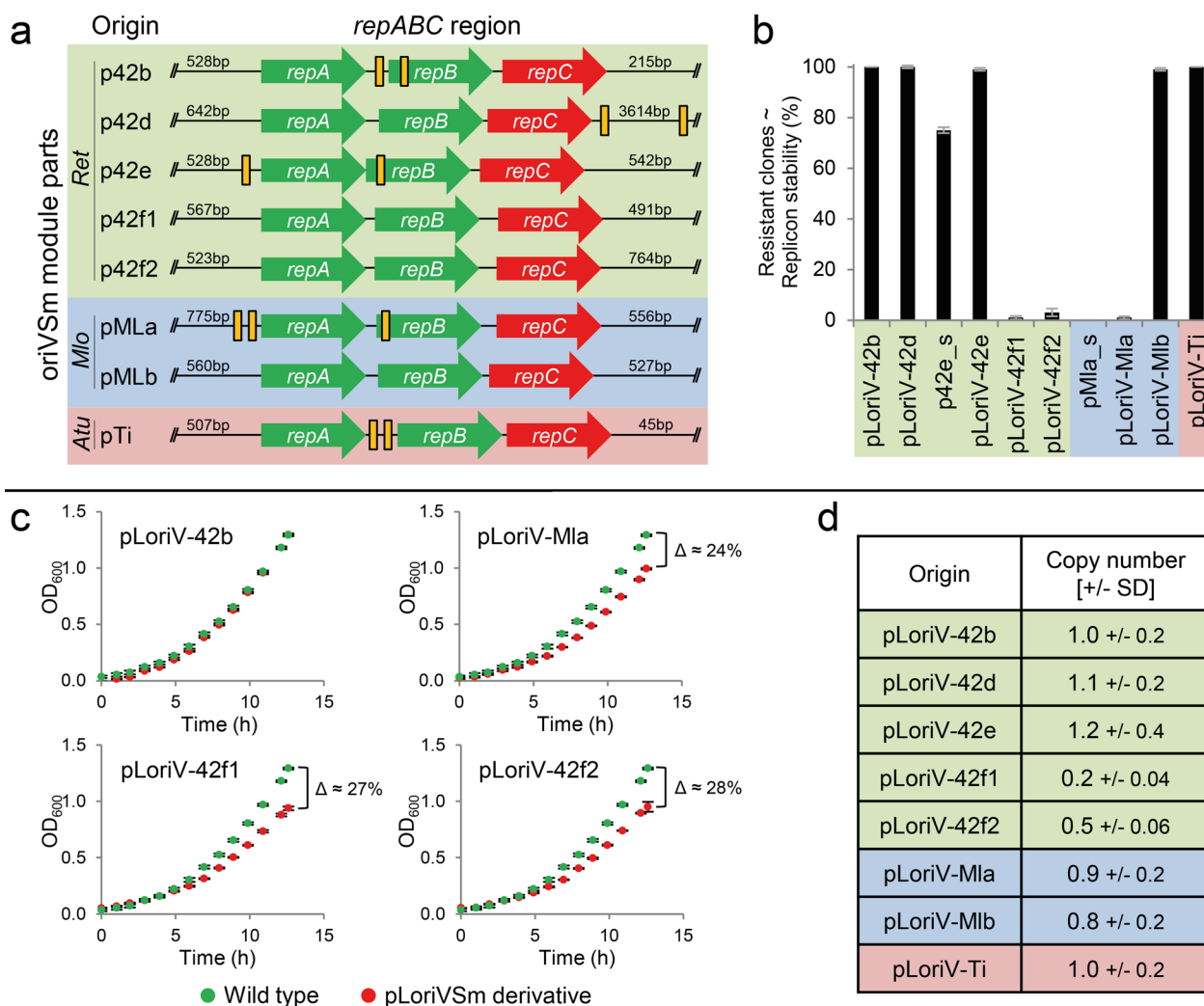


Figure 2. Characterization of oriVSm module parts. (a) *repABC* cassettes analyzed in this study. Regions are derived from *R. etli* (*Ret*), *M. loti* (*Mlo*), and *A. tumefaciens* (*Atu*) plasmids. Since functional elements, such as partitioning sites (*repS*, yellow boxes) or the promoter of the antisense RNA *inca* 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* CFN101,⁷⁷ 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, 42e, -Mla, -Mlb, 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 ~0.05. Single colonies were examined for antibiotic resistance ($n = 100$ for pLoriVSm derivatives; $n = 50$ for p42e_s and pMla_s). 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^r). 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 (-42b, -42d, -42e, -42f1, -42f2, -Mla, -Mlb, and -Ti) in exponentially growing *S. meliloti*. Measurements are based on eight technical replicates from two independent clones (see Table S6). SD: standard deviation.

to pABC vectors. LCR was shown to efficiently assemble up to ten 2 kb DNA fragments.⁴⁷ 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* DH5 α 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>).⁴⁰ 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, -42e, -42f1, -42f2), *M. loti* pMla,b (pLoriV-Mla, -Mlb), and *A. tumefaciens* pTi (pLoriV-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

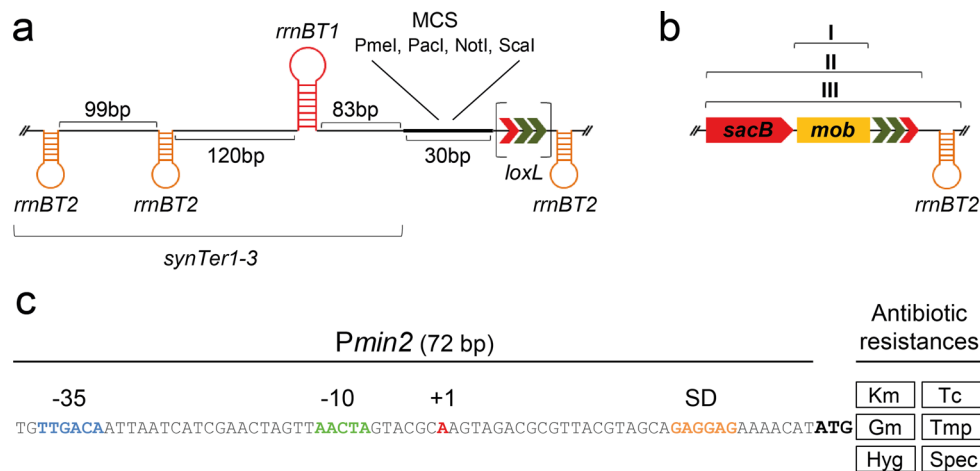


Figure 3. Setup of pABC library modules. (a) The *synTer*-MCS module plasmids provide three \sim 550 bp *synTer* regions composed of Rho-independent *E. coli* terminators *rrnBT1* (90 bp) and *rrnBT2* (60 bp) separated by unique synthetic sequences. Terminator sites (loop structures) are intended to protect the *oriVSm* module and the MCS, which provides unique recognition sites for rare cutters. pLsynTerX-*lox* plasmids are further equipped with a left arm mutated *loxL* site (arrows in brackets). (b) The *oriT* module comprises a 464 bp DNA fragment containing the RK2-based *mob* site (I). For Cre/*lox* applications the region is extended by *sacB-loxR* (II) and a further *rrnBT2* transcription terminator (III). (c) Module AR contains the short *Pmin2* fragment conferring resistance to kanamycin (Km), tetracycline (Tc), gentamicin (Gm), trimethoprim (Tmp), hygromycin (Hyg), or spectinomycin (Spec) in *E. coli* and *S. meliloti*.

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*_{p42e} 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_s) 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 *oriV*_{p42f1}, *oriV*_{p42f2}, and *oriV*_{pMla} derived cassettes were virtually lost in the same period of time. Expanding the 3'UTR of *repABC*_{pMla} from 45 bp (pMla_s) to 556 bp (pLoriV-Mla) did not improve plasmid maintenance. Slight incompatibility between *repABC*_{pMla} 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, pMlb, 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*_{pSymA}, which 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 qPCR-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 PmeI, 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 *oriV*_{pMB1}) failed. For this reason, *oriVEc* parts harboring *oriV*_{pMB1}, *oriV*_{p15A}, and *oriV*_{pSC101*} 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 bacteria⁴⁸ including *S. meliloti*. This module is available on the pLoriT library plasmids (Table 1).

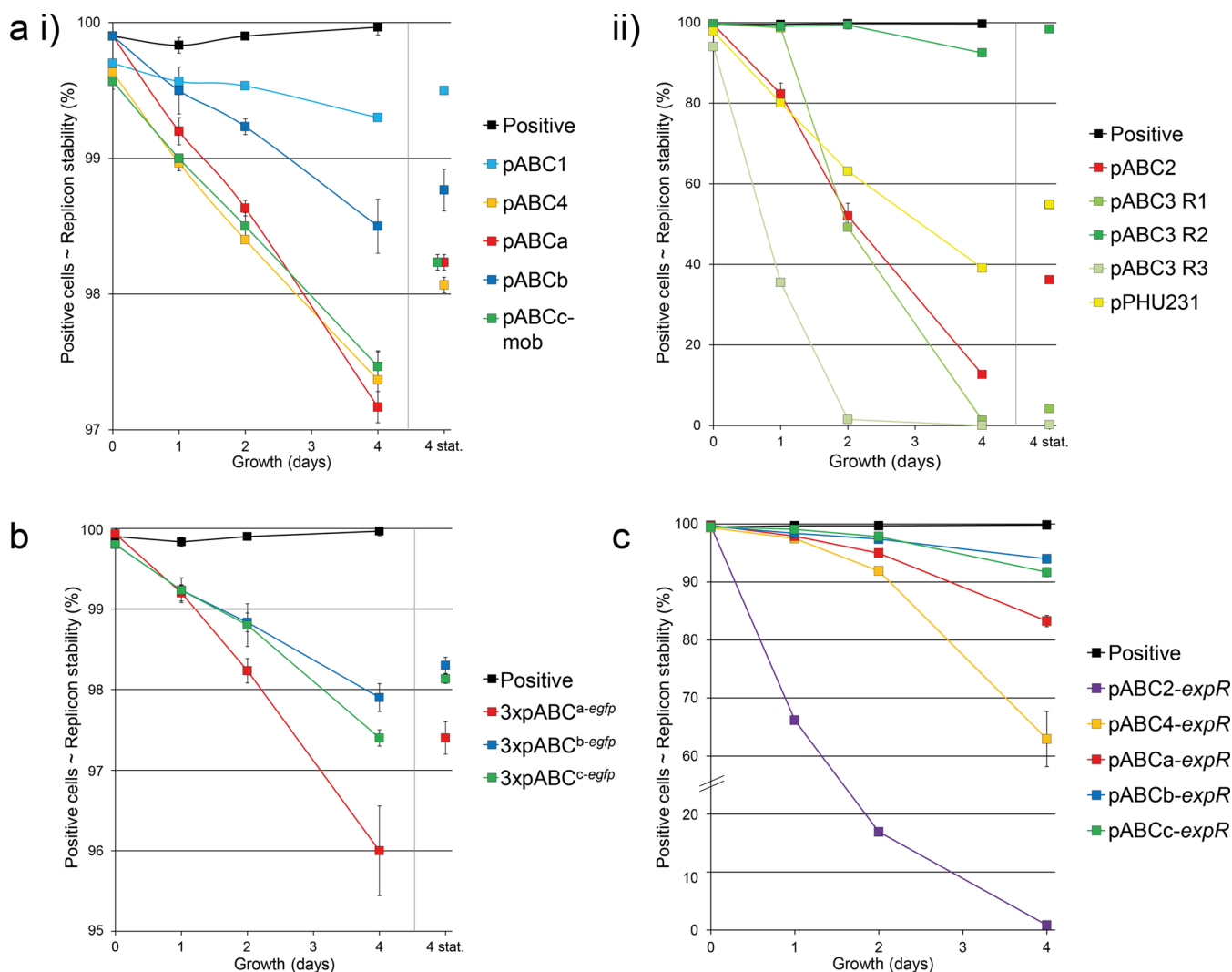


Figure 4. Inheritance stability of pABCs in *S. meliloti* Rm1021. *S. meliloti* carrying pABC-egfp (-expR) derivatives were grown in nonselective TY medium at 30 °C for 96 h. During the time course, cultures were diluted every 12 h to an OD₆₀₀ of ~0.05. An eGFP signal of 10,000 cells was measured by flow cytometry at time point 0 and after 1, 2, and 4 days. Additionally, after the initial 24 h, subcultures were incubated for 48 h until the stationary phase was reached, and reinoculated at day 3 (4 stat., separated by a line). Varying ordinate labeling should be noted. Positive: *S. meliloti* MWSm37 (genomically integrated *egfp*). Error bars indicate standard deviation calculated from three biological replicates. (a) Maintenance of single pABC-egfp derivatives. (i) Stable pABC-egfp constructs which did not exceed 97% propagation stability. (ii) Replicon stability of unstable pABC-egfp derivatives and low copy plasmid pPHU231-egfp. Because of the heterogeneous behavior of pABC3-egfp, measurements from individual clones are shown (R1–3). (b) Replicon stability of pABCs in a triple pABC system. 3xpABC^{a-egfp}: pABCa-egfp, pABCb, pABCc-mob; 3xpABC^{b-egfp}: pABCa, pABCb-egfp, pABCc-mob; 3xpABC^{c-egfp}: pABCa, pABCb, pABCc-mob-egfp. (c) *S. meliloti* carrying pABC-egfp-expR derivatives.

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 SmCre Δ hsdR 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). pABCa-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 pABCc 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 pLoriVSm library plasmids (Figure 2b), which tends to pre-select 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 OD₆₀₀ of 0.05 to 1.0.

Consistent with the properties of the corresponding pLoriVSm plasmids, derivatives of pABCa (*oriV_{pMlb}*), pABCb (*oriV_{p42b}*), pABCc-mob (*oriV_{p42d}*), pABC1 (*oriV_{p42b}*), and pABC4 (*oriV_{p42e}*) 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 (*oriV_{pMla}*) and the low-copy plasmid pPHU231 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 *oriV_{pTi}*-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 *oriV_{pTi}* as unsuitable part for construction of stably propagating single copy pABC vectors in *S. meliloti*. The pABC maintenance assay identified *oriV_{pMlb}*, *oriV_{p42b}*, *oriV_{p42d}* and *oriV_{p42e}* as highly suitable origins of replication for generation of *S. meliloti* pABC vectors.

To rule out mutual influences of the *repABC* cassettes in multi-pABC setups we further studied replicon stability in *S. meliloti* strains simultaneously carrying two or three pABCs. pABCa–pABCc retained highly stable propagation (>96%) throughout the time course and achieved stability values which were only marginally lower as compared to that of the strains carrying only one of these pABCs (Figure 4b). pABC3 and pABC4 were combined to study the influence of an instably propagating pABC on maintenance of a stably inherited pABC. In combination, pABC3 and pABC4 showed the same properties as each did individually; namely, biological replicates show great variation in the maintenance of pABC3, while pABC4 was stably inherited (Figure S10). In accordance with the previous growth studies of strains harboring stably propagating pLoriVSm plasmids (Figure 2c and S2), the corresponding pABCs did not affect *S. meliloti* cell growth even when present in combination (Figure S11).

We further tested maintenance of stably propagating pABCs under more challenging conditions by loading pABC vectors with the *expR* gene, whose presence restrains growth of

S. meliloti.⁵⁰ In the reference strain Rm1021, *expR* is disrupted by an insertion element.⁵¹ Complementation of this mutation results in a strong increase in galactoglucan production since the LuxR-type regulator ExpR is a key activator of biosynthesis of these exopolysaccharides.^{51–55} *expR* under the control of *PlacZ* 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,c, 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* cassettes derived 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 *parS*/mChr-ParB⁵⁶ and *tetO*/TetR-YFP⁵⁷ pairs were applied as a taurine-inducible dual color label system. Heterologously expressed DNA-binding proteins mChr-ParB and TetR-YFP bind to *parS* and *tetO* sequences, respectively, allowing for specific labeling of individual pABCs. Localization patterns of *parS*/mChr-ParB and *tetO*/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 pLoriVSm constructs determined by qPCR (Figure 2d). Cell populations of the *S. meliloti* strains with the unreliably inherited pABC2 (*oriV_{pMla}*), pABC5 (*oriV_{p42fi}*) and pABC6 (*oriV_{p42f2}*) 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*.

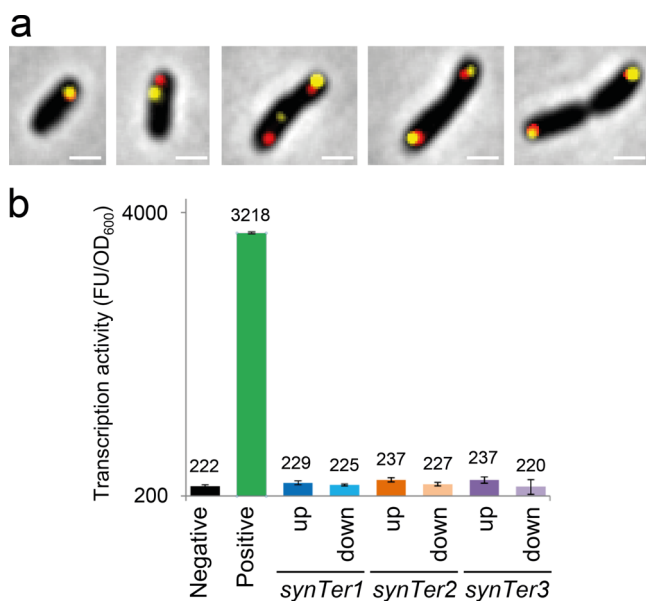


Figure 5. Examination of pABCs in *S. meliloti*. (a) Fluorescence microscopy of *S. meliloti* JDSm106 carrying pABCa-*tetO*, pABCb, and pABCc-*parS*_{Yp}. Expression of reporters *tetR-YFP* (yellow) and *mChr-parB*_{Yp} (red) was induced with 15 mM taurine for 4 h. Snapshots show cells at different stages of the cell cycle. Scale bar: 1 μ m. (b) eGFP fluorescence of *S. meliloti* Rm1021 carrying pABC1-*egfp_f/r* (*synTer1*), pABC1-ST2-*egfp_f/r* (*synTer2*), and pABC1-ST3-*egfp_f/r* (*synTer3*) was taken as a measure for the transcription activity in the MCS of library-derived pABCs. Promoter-less *egfp_f/r* 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.

repABC Mediated in Vivo Cloning in *S. meliloti* (ABC Cloning). Several methods have been developed which enable direct cloning of large gene clusters, including transformation-associated recombination (TAR) in *Saccharomyces cerevisiae*,^{59–62} RecET-mediated linear-plus-linear homologous recombination (LLHR) in *E. coli*,⁶³ and ϕ BT1 integrase-mediated site-specific recombination in *Streptomyces* species.⁶⁴ The Cre/*loxP* plus BAC strategy⁶⁵ and *oriT*-directed cloning⁶⁶ are well established *in vivo* approaches which facilitate cloning of large DNA fragments derived from α -rhizobia. The former strategy is based on Cre/*loxP*-mediated circularization of the DNA fragment of interest bearing a BAC *oriV*, which is purified from the crude cell culture and introduced to *E. coli* by electroporation.⁶⁵ The latter strategy involves a Flp-mediated excision of the FRT site bordered DNA fragments including an *oriT* site and an *E. coli* plasmid *oriV*, instantly followed by mobilization of the excised DNA to *E. coli*.⁶⁶ These direct cloning methods have proven to be very useful. However, they do not allow reliable maintenance of excised DNA in propagating donor cells, but require either DNA purification from treated cells and subsequent transformation of the crude mix into a suitable host or excision and transfer in a single step and therefore entail reduced efficiency and increased screening efforts. Properties of α -rhizobial megaplasmids such as single copy number, faithful inheritance to daughter cells, and large size make *repABC*-type *oriVs* ideal tools for *in vivo* cloning strategies. We devised an *in vivo* cloning strategy involving Cre/*lox* mediated excision of the target region, followed by stable extrachromosomal establishment of the excised region on

an autonomously replicating *repABC*-type replicon, and demonstrated its feasibility by translocation of the \sim 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*_{pMlb} 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*_{pMlb}-*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 pSRKGm 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*_{pMlb}-*lacO*.

Figure 6b,i depicts the genomic situation of *S. meliloti* SmCre-*exo*-IN bearing pIso 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*, pIso provides a *loxL* site which is preceded by a terminator sequence preventing transcription of the promoter-less spectinomycin resistance gene *aadA1*. pIso further offers a constitutively expressed *lacI* gene encoding the transcriptional repressor which mutes the adjacent *repABC*_{pMlb}-*lacO* operon in the absence of IPTG. This repression of *oriV* activity enables integration of pIso into the genome. Accordingly, the efficiency of *S. meliloti* transformation with pIso was \sim 4000-fold decreased when mating mixtures were incubated on TY agar lacking IPTG and \sim 20% of transconjugants carried the construct integrated into the genome (Figure S15a). Induction of Cre expression and simultaneous activation of *oriV*_{pMlb-lacO} by IPTG resulted in excision and maintenance of the floxed DNA fragment, respectively, giving rise to strain *S. meliloti* SmCre-*exo*-OUT carrying pIso-*exo* as an autonomous mini-replicon. Successful Cre-mediated recombination, resulting in an active *loxP* site on pIso-*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 \sim 75% after Cre/*lox*-mediated isolation, which can be attributed to highly efficient Cre recombination in SmCre derivatives⁴⁵ in general, and suitable function of the engineered *repABC*-type *oriV* in particular. Since multiple *lox* sites recombine in an unpredictable manner, a single copy number of activated *repABC*_{pMlb}-*lacO* plasmids is an important prerequisite for manageable Cre reactions. Promoter modification appeared to affect the propagation stability of pMlb-*lacO* and pIso-*exo*, while retaining copy number control, as suggested by the qPCR-based copy number determination (Figure S15c).

Transfer of pIso-*exo* to *E. coli* was achieved via triparental mating. *oriV*_{pMB1} on the pIso backbone enabled replication in *E. coli* due to the high copy number efficient purification of the large DNA molecule from this host (Figure 6b,ii). 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

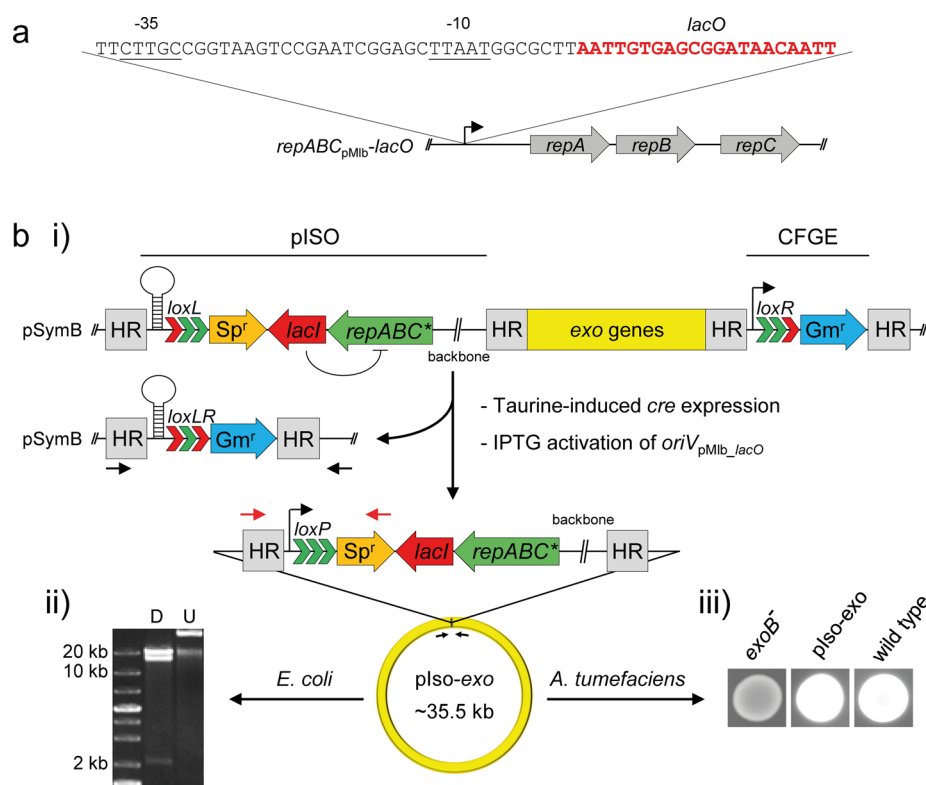


Figure 6. *repABC* mediated *in vivo* cloning in *S. meliloti* (ABC cloning). (a) The promoter region of *repABC*_{pMLb} was predicted according to the consensus motifs of *S. meliloti* σ^{70} promoters (−35 and −10 elements are underlined). A single *lacO* box (red letters) was integrated at the predicted transcription start site (+1). (b) ABC cloning of the *exo* gene cluster in *S. meliloti* SmCre. Three consecutive arrow heads indicate *lox* sites with native (green) or mutated arms (red). (i) A gentamicin resistance cassette carrying a right arm-mutated *loxR* site (*Pmin2-loxR-aacC1*) was integrated downstream of the gene cluster via cloning-free genome editing⁴⁵ (CFGE). Subsequently, pISO bearing *repABC*_{pMLb}-*lacO* (*repABC*^{*}) was integrated via a ~550 bp homologous region (HR) upstream of the gene cluster, resulting in *S. meliloti* SmCre_{exo}-IN. Taurine induction of Cre expression and simultaneous IPTG activation of *oriV*_{pMLb_lacO} gave rise to SmCre_{exo}-OUT which carries the relocated ~35.5 kb region (pIso-exo) comprising the entire *exo* gene cluster. Both the deletion site on pSymB and the fusion site on pIso-exo were PCR-amplified with primers 94 + Rev (black arrows) and 680 + 128 (red arrows), respectively, and sequencing of PCR products confirmed proper Cre-mediated recombination. (ii) pISO-*exo* was transferred to *E. coli* via triparental mating, resulting in *E. coli*/pIso-*exo*. Purified plasmid DNA was digested with *NheI*, resulting in fragments of 2.03 kb, 13.8 kb, and 19.6 kb fragments (D). Sequencing of the fusion site comprising *loxP* and *aadA1* with primers 680 and 128 (red arrows) further confirmed successful cloning. U: Undigested plasmid DNA. (iii) Calcofluor fluorescence assay. pISO-*exo* was transferred to the exopolysaccharide-deficient *A. tumefaciens* C58 *exoB* mutant (*exoB*⁻) via triparental mating, giving rise to *A. tumefaciens* *exoB*/pIso-*exo* complemented for exopolysaccharide production (pIso-*exo*), and thus showing UV-induced fluorescence on Calcofluor-containing medium. Wild type: *A. tumefaciens* C58.

heterologous *repABC*-based vectors beyond *S. meliloti*, a pilot survey was performed in *Agrobacterium tumefaciens* C58, *Mesorhizobium loti* MAFF303099, *Sinorhizobium medicae* CC169, *Sinorhizobium fredii* NGR234, *Rhizobium leguminosarum* Norway, *Methylobacterium extorquens* AM1 Δ *cel*, and *Caulobacter crescentus* CB15N (Table S9). In the diverse tested members of the *Rhizobiales*, 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*_{pMLb}) 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 probably applicable in the *Rhizobiales* beyond the *Rhizobiaceae*, particularly considering the possibility of further expanding the portfolio of *repABC* modules in the future.

Conclusions. The currently available ectopic expression systems applicable in *S. meliloti* and related bacteria are based on a small number of low-copy and multicopy vectors.

Because of incompatibilities, options for combining different vectors in one cell are very limited. Moreover, different copy 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 plasmids²³ 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*_{repABC}-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*.⁶⁷ 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 mutants⁴⁵ 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 Δ cel, *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 on peptone-yeast extract (PYE) medium.⁷⁰ If necessary, the following antibiotics were used: gentamicin (8 μ g/mL for *E. coli*, 30 μ g/mL for *S. meliloti*, and 50 μ g/mL for *A. tumefaciens*), kanamycin (25 μ g/mL for *C. crescentus*, 30 μ g/mL for *S. fredii*, and *R. leguminosarum*, 50 μ g/mL for *E. coli*, *M. loti*, and *M. extorquens*, 70 μ g/mL for *A. tumefaciens*, 200 μ g/mL for *S. meliloti*), streptomycin (600 μ g/mL for *S. meliloti* and *R. leguminosarum*), spectinomycin (100 μ g/mL for *E. coli*, 200 μ g/mL for *S. meliloti*, and 500 μ g/mL for *A. tumefaciens*), hygromycin (100 μ g/mL for both *E. coli* and *S. meliloti*), nalidixic acid (10 μ g/mL for *A. tumefaciens*, *S. fredii*, *M. loti*, and *S. medicae*), trimethoprim (1000 μ g/mL for *E. coli*, 700 μ g/mL for *S. meliloti*), chloramphenicol (20 μ g/mL for *E. coli*) or tetracycline (10 μ g/mL for *E. coli* and 3 μ g/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* C58 *exoB* carrying *pIso-exo* were performed on LB agar supplemented with gentamicin and spectinomycin, 200 μ g/mL calcofluor, and 500 μ M 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.⁶⁸ 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.⁴⁵ Preparation of electrocompetent cells was done as described by Ferri 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 ligase 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-eYFP* (derived from the fluorescent repressor operator system, FROS⁵⁷) and *mChr-parB_{Yp}* (*parB* derived from *Yersinia pestis*) was developed. Homologous regions comprising the upstream region of *tauA* (pSymB position 1050453–1050952) and *PtauAB-tauA* (pSymB position 1050781–1051458) were integrated into pK18mobsacB, thereby flanking *tetR-YFP-mChr-parB_{Yp}*. *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-mChr-parB_{Yp}* reporter construct stably integrated into the chromosome downstream of the *tauA* promoter. Localization studies of pABCs carrying either a pLAU44-derived *tetO* array⁵⁷ (position 5117-9290) or a pMT1-derived, 86 bp *parS_{Yp}* sequence⁷¹ 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 *Pmin2-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* Δ hsdR 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 *Pmin2-loxR-aacC1* (PCR amplified from pJD211 with primers Uni/Rev) was blunt-end ligated with a ~550 bp fragment covering the *exoP-thiD* intergenic region (pSymB position 1190665–1191220; PCR amplified with primers 86/87). Purified ligation products were then introduced to SmCre Δ hsdR by electroporation. Finally, a transformant exhibiting *Pmin2-loxR-aacC1* correctly integrated into the genome downstream of the *exo* gene cluster was transformed with pIso, giving rise to strain SmCre_exo-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* cells 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 pISO-*exo* from *S. meliloti* into different species, triparental matings between *S. meliloti* SmCre_exo-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 an OD₆₀₀ of 2.0 and equal volumes of donor, recipient, and helper strain were mixed. A 50 μ L aliquot of cell suspension was dropped on LB agar supplemented with 500 μ M IPTG 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 suspensions was adjusted to 2.0 and appropriate dilutions were plated on selective LB agar containing 500 μ M 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 CFU 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 emerging colonies were streaked on both selective (Km) and nonselective TY agar. Antibiotic resistance was correlated with the presence of the assessed plasmid.

For determination of propagation stability of pABC-*egfp* derivatives, *S. meliloti* cultures were incubated over 96 h at 30 °C in TY medium. Probes were taken 0, 24, 48, and 96 h postinoculation and immediately diluted to an OD₆₀₀ of 0.1 in 0.9% (w/v) NaCl. eGFP fluorescence was detected using a BD Fortessa flow cytometer. For FACS analysis 10,000 gated events were acquired giving rise to the ratio between eGFP-negative and positive cells. Sufficient sensitivity was verified (Figure S9).

qPCR-Based Copy Number Determination. *S. meliloti* cultures were grown in selective TY medium until OD₆₀₀~1.0 was reached and adjusted to $\sim 1.39 \times 10^5$ cells/ μ L (Δ 1 ng DNA/ μ L). Cell suspensions that had been incubated at 95 °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 TaykonNo Rox SYBRMasterMix dTTP Blue Kit. Reactions were performed according to the manufacturer's instructions in a 5 μ L 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 multiline Argonlaser (65 mW; Melles Griot) were applied with AHF 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 Designer⁷⁶ 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*_{pSymA} 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 SmCre Δ hsdR. 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 pABCa-c. Figure S15. Examination of ABC cloning procedures (PDF)

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Author Contributions

#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 assays of *repABC*-based plasmids in diverse bacteria. P.S. computationally analyzed parts for sequence similarities. J.D. and M.W. analyzed all data. J.D. and A.B. wrote the paper.

Notes

The authors declare no competing financial interest.

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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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