nature biotechnology

A robust system for production of minicircle DNA vectors

Mark A Kay[1,2], Cheng-Yi He[1,2] & Zhi-Ying Chen[1,2]

Minicircle DNA vectors allow sustained transgene expression in quiescent cells and tissues. To improve minicircle production, we genetically modified *Escherichia coli* to construct a producer strain that stably expresses a set of inducible minicircle-assembly enzymes, ΦC31 integrase and I-SceI homing endonuclease. This bacterial strain produces purified minicircles in a time frame and quantity similar to those of routine plasmid DNA preparation, making it feasible to use minicircles in place of plasmids in mammalian transgene expression studies.

A minicircle episomal DNA vector is a circular expression cassette devoid of the bacterial plasmid DNA backbone. These vectors have been used for years in preclinical gene transfer research because of their 10- to 1,000-fold enhancement compared with regular plasmids in long-term transgene expression in quiescent tissues *in vivo*[1,2] and *in vitro*[3]. The mechanism of enhanced transgene expression is unclear but may result from eliminating heterochromatin formation induced by the plasmid backbone[4] and/or from reducing the death of transfected cells from inflammation due to CpG responses when plasmids are delivered by lipid carriers[5].

The major obstacle to widespread use of minicircles has been their time-consuming, labor-intensive production. In our previous minicircle production schemes (**Fig. 1a**), the minicircle producer plasmid contained a transgene expression cassette flanked with attB and attP, a set of inducible enzyme genes (a gene encoding homing endonuclease I-SceI and two copies of the gene encoding ΦC31 integrase) and an I-SceI recognition site[6]. The attB and attP sites are the bacterial and phage attachment sites of ΦC31 integrase, and the ΦC31 and I-SceI genes are regulated by the L-arabinose–inducible araCBAD system. Minicircle DNA is generated by recombination between the attB and attP sites, and I-SceI initiates the destruction of the plasmid DNA backbone circle by cutting through the engineered I-SceI site (**Fig. 1a**). Although the yields from this protocol were ~1 mg of minicircle DNA from 1 liter of overnight culture, the preparations still contained ~3–15% of the input minicircle producer plasmid plus the plasmid backbone circle as contaminants. Including CsCl equilibrium gradient centrifugation to remove these unwanted DNAs, the production procedure is four labor-intensive days longer than routine plasmid production protocols. Other groups have made minicircle DNA vectors using different recombinases, such as the bacteriophage λ integrase[7] or Cre recombinase[8]. Limitations of these approaches include low yields, high contamination or the need for expensive, labor-intensive techniques to isolate minicircles from bacterial lysates[9].

We present a system that allows simple, rapid and inexpensive production of a high-quality form of minicircles (**Fig. 1b**,**c**). We reasoned that the impurity plasmid DNAs in our earlier minicircle production schemes (**Fig. 1a**) resulted largely from an all-or-none phenomenon[10], such that in a subset of bacterial cells, the L-arabinose transporter AraE is not expressed, resulting in the failure of ΦC31 integrase–directed formation of minicircle and I-SceI–mediated degradation of the plasmid backbone circle. To overcome this limitation, we replaced the *E. coli* 'Top10' cells with the BW27783 bacterial strain[10], in which the araE gene was driven by the constitutive promoter, cp8. When used in combination with previous minicircle producer plasmids, the conversion to minicircle DNA was more complete (**Fig. 2**) even with the addition of a very small amount (0.001%) of L-arabinose to the culture (**Fig. 2a**). There was variable but substantial plasmid DNA degradation in these preparations (**Fig. 2a**,**c**). We hypothesized this was due to the presence of endonuclease A, which was confirmed when we knocked the gene out (**Fig. 2b**,**c**), resulting in strain BWΔendA (**Fig. 2b** and **Supplementary Fig. 1**).

Trace amounts of impurity DNAs became visible after knocking out the gene encoding endonuclease A (**Fig. 2c**), suggesting that there was still a lack of complete recombination after L-arabinose induction. Thus, we engineered the bacterial cells to simultaneously express a second L-arabinose transporter, LacY A177C[11] and created the strain 2T (**Supplementary Figs. 1** and **2a–d**). Its wild-type counterpart, LacY, is a glucose transporter, which gains an L-arabinose transporter function with the described missense mutation. This further reduced but did not completely eliminate the contaminating DNAs (data not shown).

To eliminate any possible contamination of the genes encoding ΦC31 integrase and I-SceI from the small amount of parental plasmids used to generate minicircle DNAs, we relocated both genes from the minicircle producer plasmid to the bacterial genome. Accordingly, we integrated three copies of the BAD.I-SceI cassette using the bacteriophage λ Red homologous recombination system[12] and created the strain 3S2T (**Supplementary Figs. 1** and **3a**). We found that the integrated BAD.I-SceI was fully functional, as evident by the efficient destruction of a plasmid with eight consecutive I-SceI sites after L-arabinose induction (**Supplementary Fig. 3b**,**c**).

Integration of the BAD.ΦC31 gene was less straightforward. First, the BAD.ΦC31 cassette could not be integrated using our original Red system without destroying the integrated BAD.I-SceI gene. Second, we attempted to integrate the BAD.ΦC31 cassette from a plasmid through ΦC31 integrase–mediated recombination between the attB site in the plasmid and attP pre-integrated into the genome. However, this integrant was unstable (data not shown), most likely because of a reverse

[1]Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, California, USA. [2]The Center for Clinical Science Research, Stanford University School of Medicine, Stanford, California, USA. Correspondence should be addressed to M.A.K. (markay@stanford.edu) or Z.-Y.C. (zychen98@stanford.edu).

Received 21 June; accepted 15 October; published online 21 November 2010; doi:10.1038/nbt.1708

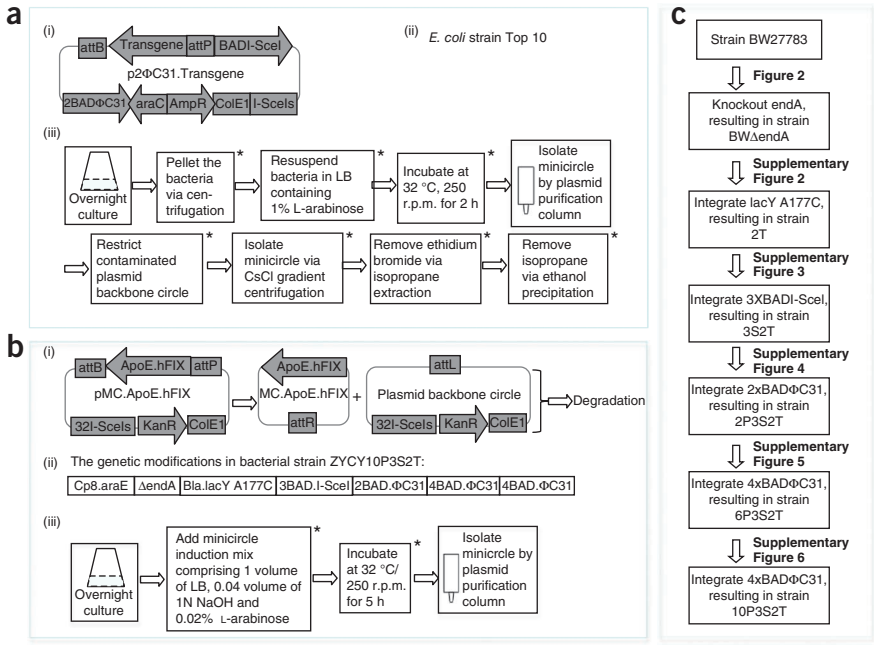


Figure 1 Comparison of the present and previous minicircle systems. (**a**) An earlier version of the minicircle production system. (i) Structure of the previous minicircle producer plasmid. BAD and araC, the promoter and the repressor gene of the inducible L-arabinose-araC.BAD system; ΦC31, bacteriophage ΦC31 integrase gene; attB and attP, the bacterial and phage attachment sites of the ΦC31 integrase; I-SceI, I-SceI homing endonuclease gene; I-SceIs, the I-SceI recognition site; AmpR, ampicillin resistance gene; ColE1, DNA replication origin. (ii) *E. coli* strain Top10, original strain used to produce minicircle. (iii) Flow chart showing the minicircle production protocol. Each box represents a major step and the starred boxes represent the steps required in addition to a routine plasmid production protocol. (**b**) The present minicircle system. (i) Diagram of a minicircle producer plasmid and its conversion to minicircle DNA. pMC.ApoE.hFIX, minicircle producer plasmid; hFIX, human factor IX; sApoE, promoter/enhancer, as described previously[1]; KanR, kanamycin resistance gene. Upon L-arabinose induction, ΦC31 is expressed to mediate the formation of minicircle and plasmid backbone circle and I-SceI to induce the destruction of plasmid backbone circle. (ii) The genetic modifications of the minicircle producing bacterial strain ZYCY10P3S2T. 10P3S2T stands for (1) ten copies of BAD.ΦC31 cassette, that were integrated in three loci of the bacterial genome: two tandem copies at the ΔendA locus (**Supplementary Fig. 4b**), and four copies at the araD (**Supplementary Fig. 5a**) and galK (**Supplementary Fig. 6a**) each; (2) three tandem copies of BAD.I-SceI cassette, which were integrated at UMU locus (**Supplementary Fig. 3a**) and (3) two genes constitutively expressing L-arabinose transporter, one was araE gene driven by an artificial promoter cp8, which presented in strain BW27783 (ref. 10); the other was the bla-lacY A177C cassette, which was integrated at the lacY locus (**Supplementary Fig. 2**); bla, beta-galactosidase gene promoter; lacY A177C, the missense mutant of lacY gene. (iii) Flow chart showing the present minicircle production protocol. (**c**) Stepwise genetic modification of the bacterial genome to make the current ZYCY10P3S2T strain.

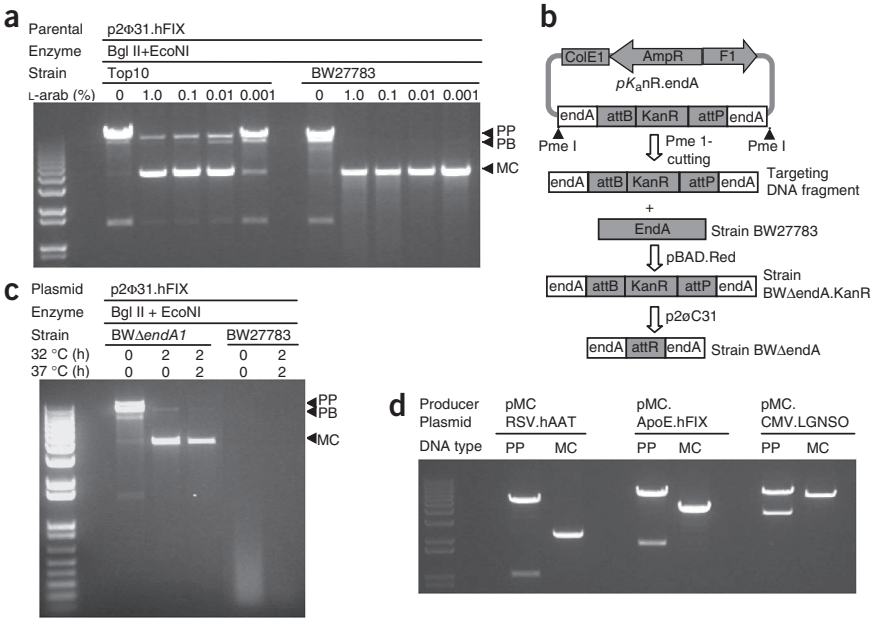
reaction mediated by leaky ΦC31 integrase expression in concert with an uncharacterized cofactor[13]. Third, because many more copies of the BAD.ΦC31 were needed to efficiently generate minicircles, we designed a strategy using ΦC31 integrase and a second recombinase. After making the desired integrant by the ΦC31 integrase–mediated reaction, the resulting attL site was removed by the second recombinase, eliminating the possibility of the reverse excision reaction (**Supplementary Figs. 4a–c**, **5a**,**e** and **6a**,**d**).

Using the above approach, we generated a bacterial host containing two copies of the BAD.ΦC31 inserted into the deleted endA site (ΔendA), called the 2P3S2T strain (**Supplementary Figs. 1** and **4a–c**). We found that ΦC31 integrase mediated the formation of the minicircle, and I-SceI mediated destruction of the unrecombined minicircle producer plasmid and plasmid backbone circle in a minicircle producer plasmid encoding a 2.2-kb transgene and 32 tandem copies of the I-SceI site in the plasmid backbone (**Supplementary Fig. 4d**,**e**). However, the minicircle yield was low, suggesting that most of the minicircle producer plasmid was destroyed by I-SceI before minicircle formation, and that additional ΦC31 integrase activity was needed to mediate greater minicircle formation.

We therefore used another targeting plasmid to integrate four copies of the BAD.ΦC31 cassette into the araD locus (**Supplementary Figs. 1** and **5**), and four additional copies into the gene encoding galK (**Supplementary Figs. 1** and **6**), resulting in strains 6P3S2T and ZYCY10P3S2T carrying six and ten copies of BAD.ΦC31, respectively. These two present strains, in concert with the minicircle producer plasmid described earlier (**Supplementary Fig. 4d**), enabled three improvements over the previous system (**Fig. 1a**)[6]. First, the procedure was greatly simplified and, compared to a routine plasmid preparation, required only an additional temperature change and 5-h incubation after addition of L-arabinose (**Fig. 1b**). Second, the yield of three minicircles, with a size range of 2.2–6.0 kb, was 3.4–4.8 mg/1,000 ml of overnight culture, making it ~3- to 5-times higher (**Fig. 2e**) than our previous minicircle producing system (**Fig. 1a**). In addition, compared with the previous minicircle production protocol, there were 10-times fewer contaminating plasmid DNAs, ranging from 0.4% to 1.5% of the input minicircle preparation as determined by qPCR (Online Methods, qPCR section)[6]. On a molar scale, the yield of minicircle was 20–70% higher than the minicircle producer plasmid (**Fig. 2e**). Third, the cost of minicircle production was similar to that of a standard plasmid.

These production improvements, together with their superior expression profiles, make it feasible for minicircle DNA vectors to be used in place of plasmid DNAs in mammalian expression studies.

Our efforts to optimize minicircle production led us to develop an improved bacterial genome modification strategy. The enhancements include: (i) the use of a circular integrating plasmid instead of linear DNA, allowing repeated integration of the same or different DNA sequences of up to 10 kb in selected targets (**Supplementary Figs. 4b**, **5a** and **6a**); (ii) inclusion of a second recombinase, allowing selective removal of unwanted sequences, such as the hybrid sequences responsible for the reverse recombinase reaction, along with the useless or harmful plasmid backbone DNAs, from the genome (**Supplementary Figs. 4b**, **5a** and **6a**); and (iii) the use of the TPin/9attB.9attP recombination system (**Supplementary Figs. 5a** and **6a**), eliminating the inherent problems with the FLP/FRT system, in which the uncontrolled reaction between the substrate and product FRTs makes repeated integration virtually impossible. Although these bacterial gene modifications would be difficult to achieve with conventional homologous recombination methods, there are other technologies using dual site-specific recombinases for targeted integration of circular DNA into the bacterial



e

Minicircle producer plasmid	Size (kb)		Yield (mg/liter)		Weight ratio (MC/PP)	Yield (E-9 mole/liter)		Molar ratio (MC/PP)
	PP	MC	PP	MC		PP	MC	
pMC.RSV.hAAT	6.1	2.2	5.84 ± 0.41	3.42 ± 0.87(I)	0.59	1.60 ± 0.11	2.59 ± 0.66(I)	1.63
pMC.ApoE.hFIX	8.1	4.2	5.45 ± 0.38	4.83 ± 0.60(II)	0.89	1.12 ± 0.08	1.91 ± 0.24(I)	1.71
pMC.CMV.LGNSO	9.8	6.0	5.57 ± 0.15	3.40 ± 0.35(I)	0.61	0.95 ± 0.03	1.07 ± 0.11(II)	1.18

Figure 2 Improvement in minicircle quality and quantity. (**a**) Strain BW27783 produced minicircle (MC) with enhanced purity. Minicircles were produced according to the protocol described previously[6]. 2Φ31.hFIX, minicircle producer plasmid (PP); BglII+EcoN1, two restriction enzymes used to cleave MC before electrophoresis; L-arab(%), percent of L-arabinose in the minicircle induction reaction; PB, plasmid backbone circle. (**b**) Strategy for inactivation of endA. pK_anR.endA, the plasmid used to generate the Pme1-restricted targeting DNA fragment; KanR, kanamycin-resistance gene; attB and attP, the bacterial and phage attachment sites of bacteriophage ΦC31 integrase; boxed endA, PCR-generated 329- and 754-bp end A fragments; pBAD.Red, a plasmid expressing the bacteriophage λ homology recombination complex (Red) under the control of araC.BAD (BAD); p2ΦC31, a complementing plasmid encoding two copies of BAD.ΦC31 gene, one copy of BAD. I-SceI gene and one I-SceI site; BWΔendA, a strain derived from BW27783 with the endA interrupted. (**c**) Minicircle DNA integrity before and after disruption of the endA gene. Before disrupting endA, we observed repeatedly large variations in the degree of plasmid degradation as shown in **a** and **c**. Because the endonuclease A is a membrane-bound enzyme, it was possible that its membrane release and activation varied during plasmid preparation. 32 °C and 37 °C, the incubation temperature. All reactions contained 1% L-arabinose. (**d**) Quality of the minicircle determined by gel analyses. Minicircle was made according to the simplified protocol outlined in **Figure 1b** and **Supplementary Protocol**; DNAs were cleaved before electrophoresis. (**e**) Yield of minicircle producer plasmids and minicircle vector DNAs. The yield was derived from triplicate 400-ml overnight cultures; PP, minicircle producer plasmid; MC, minicircle. Wilcoxon rank sum test comparing the yield of minicircle and its minicircle producer plasmid: (I), $P < 0.05$; (II), $P > 0.05$. We used the following formula to convert the yield from mg/l to mol/l: mol/l = [yield (mg/l) × 10E-3 g/l]/[size (kb) × 1,000 × 330 × 2 g/mol], where 330 is the average molecular weight of dNTP. The minicircle producer plasmid, pMC.RSV.hAAT is schematically illustrated in **Supplementary Figure 4d**; the pMC.CMV.LGNSO is described in the Constructs section of Online Methods.

genome and creation of a marker-less bacterial strain[14]. However, in our approach we were able to remove one of the two recombination hybrids (either attL or attR; **Supplementary Figs. 5** and **6**), disabling the reverse reaction and resulting in a stable genomic insertion. Recent studies have shown that repeated gene sequences inserted into the bacterial genome are stable for at least 80 generations[15]. This type of site-specific integration will broaden the ability to make stable genetic modifications in prokaryotic and eukaryotic genomes.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

The authors would like to thank P. Valdmanis for critical review of the manuscript. This work was supported by the US National Institutes of Health - HL064274 (M.A.K.). Bacterial strain BW27783 was a gift of Jay D. Keasling of the University of California at Berkeley. Plasmid placY A177C was obtained from John E. Cronan at the University of Illinois.

AUTHOR CONTRIBUTIONS

Z.-Y.C. planned and carried out the experiments. C.-Y.H. conducted a number of the experiments. M.A.K. and Z.-Y.C. discussed and planned the experimental strategies. M.A.K. and Z.-Y.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturebiotechnology/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Chen, Z.Y., He, C.Y., Ehrhardt, A. & Kay, M.A. *Mol. Ther.* **8**, 495–500 (2003).
2. Huang, M. *et al. Circulation* **120**, S230–S237 (2009).
3. Jia, F.J. *et al. Nat. Methods* **7**, 197–199 (2010).
4. Riu, E., Chen, Z.Y., Xu, H., He, C.Y. & Kay, M.A. *Mol. Ther.* **15**, 1348–1355 (2007).
5. Tan, Y., Li, S., Pitt, B.R. & Huang, L. *Hum. Gene Ther.* **10**, 2153–2161 (1999).
6. Chen, Z.Y., He, C.Y. & Kay, M.A. *Hum. Gene Ther.* **16**, 126–131 (2005).
7. Darquet, A.M., Cameron, B., Wils, P., Scherman, D. & Crouzet, J. *Gene Ther.* **4**, 1341–1349 (1997).
8. Bigger, B.W. *et al. J. Biol. Chem.* **276**, 23018–23027 (2001).
9. Mayrhofer, P., Blaesen, M., Schleef, M. & Jechlinger, W. *J. Gene Med.* **10**, 1253–1269 (2008).
10. Khlebnikov, A., Datsenko, K.A., Skaug, T., Wanner, B.L. & Keasling, J.D. *Microbiology* **147**, 3241–3247 (2001).
11. Morgan-Kiss, R.M., Wadler, C. & Cronan, J.E. Jr. *Proc. Natl. Acad. Sci. USA* **99**, 7373–7377 (2002).
12. Yu, D. *et al. Proc. Natl. Acad. Sci. USA* **97**, 5978–5983 (2000).
13. Thorpe, H.M. & Smith, M.C. *Proc. Natl. Acad. Sci. USA* **95**, 5505–5510 (1998).
14. Minaeva, N.I. *et al. BMC Biotechnol.* **8**, 63 (2008).
15. Sallam, K.I., Tamura, N., Imoto, N. & Tamura, T. *Appl. Environ. Microbiol.* **76**, 2531–2539 (2010).

ONLINE METHODS

Materials. Bacterial strain BW27783 was a gift of J.D. Keasling[10]. Luria-Bertani broth (LB) powder was purchased from MP Biomedicals and Terrific Broth (TB) powder from Invitrogen. Plasmid DNA purification kits were from Qiagen.

Constructs. Plasmid pK_anR.endA (**Fig. 2b** and **Supplementary Glossary**) was made by inserting the following DNA elements: the kanamycin-resistance gene (*KanR*) flanked by the bacterial (attB) and phage (attP) attachment sites of the *Streptomyces* bacteriophage ΦC31 integrase and PCR-generated 329- and 734-bp fragments of endonuclease A gene (endA) into the pBlueScript (Stratagene) plasmid. The plasmid p2ΦC31 (**Fig. 2b** and **Supplementary Figs. 1**, **2b**, **3a** and **Supplementary Glossary**) was made by removing the human factor IX (hFIX) transgene and flanking attB and attP sites from p2ΦC31.hFIX[6], the BAD.I-SceI cassette and I-SceI site were retained. To make the plasmid p3BAD.I-SceI (**Supplementary Fig. 3a** and **Supplementary Glossary**), the araC repressor gene together with three tandem copies of BAD.I-SceI gene were inserted downstream of the attB site of the plasmid pK_anR.endA (**Fig. 2b** and **Supplementary Glossary**), followed by replacement of endA with the 737- and 647-bp UMU fragment generated by PCR. The plasmid pBS.8I-SceIs (**Supplementary Fig. 3b** and **Supplementary Glossary**) was constructed by inserting eight consecutive copies of the 18-bp I-SceI site into pBlueScript. The plasmids pBAD.Red (**Fig. 2b** and **Supplementary Figs. 1**, **2a**,**c**, **3a**, **4a**, **5a** and **6a** and **Supplementary Glossary**)[16] and pcI587.FLP (**Supplementary Figs. 1**, **4a**,**b** and **Supplementary Glossary**)[17], which both carry the temperature-sensitive A101 origin of replication, were obtained from the *E. coli* Genetic Resource Center of Yale University. Plasmid pFRT.KanR.attB (**Supplementary Figs. 1**, **4a** and **Supplementary Glossary**) was generated by inserting the attB site and the KanR flanked with FRT sites of flipase into pBlueScript using DNA oligonucleotides. Plasmid p2ΦC31.attP.FRT (**Supplementary Figs. 1**, **4b** and **Supplementary Glossary**) was constructed using our previous minicircle-producing plasmid p2ΦC31.hFIX[6] as starting material. The sApoE.hFIX and BAD.I-SceI cassettes and the I-SceI site were eliminated, the ColE1 origin and the AmpR were replaced with the KanR, the FRT and attP sites and the plasmid replication origin R6K plus zeocin-resistance gene (Zeo) derived from the plasmid pCpG-mcs (InvivoGen). The plasmid pMC.ApoE.hFIX (**Figs. 1b**, **2d**,**e** and **Supplementary Glossary**) was made by stepwise replacement of the AmpR and the F1 origin in the plasmid pBS.8I-SceI (**Supplementary Fig. 3b** and **Supplementary Glossary**) with KanR and sApoE.hFIX and the flanking attB and attP sites from the plasmid p2ΦC31.hFIX[6], followed by the insertion of an additional 24 consecutive I-SceI recognition sites. The plasmid pMC.CMV.LGNSO (**Fig. 2d**,**e**) was made by replacing the attB.RSV.hAAT.attP fragment in the plasmid pMC.RSV.hAAT (**Fig. 2d**,**e**, **Supplementary Fig. 4d** and **Supplementary Glossary**) with the fragment flanked with the attB and attP derived from the plasmid p2ΦC31.LGNSO, as described previously[3]. The plasmid placY.TetR (**Supplementary Figs. 1**, **2a** and **Supplementary Glossary**) was made by inserting the TetR sequence from pACYC184 (New England Biolabs) and flanked with PCR-generated 425 and 227 bp of the *z* and *a* lactose operon genes, respectively. Plasmid pbla.lacY A177C (**Supplementary Figs. 1**, **2a** and **Supplementary Glossary**) was made by inserting the beta-lactosidase gene promoter (bla) derived from pBlueScript fused with the lacY A177C cDNA derived from plasmid placY A177C obtained from J.E. Cronan[11]. Plasmid p9attP.TetR.attB (**Supplementary Figs. 5a**, **6a** and **Supplementary Glossary**) was made by replacing the *z* and *a* fragments in the plasmid placY.TetR with the attachment site (9attP) from bacteriophage TP901-1 (ref. 18) and attB. Plasmid p4ΦC31.attP.9attB (**Supplementary Figs. 5a**, **6a** and **Supplementary Glossary**) was generated by replacing the FRT and ZEO.R6K sequences of p2ΦC31.attP.FRT (**Supplementary Figs. 1**, **4b** and **Supplementary Glossary**) with the bacterial attachment site 9attB of bacteriophage TP901-1 and A101 from plasmid pBAD.Red[16], followed by insertion of two additional copies of the BAD.ΦC31 sequence.

Insertional inactivation of the endonuclease A gene in BW27783 (Fig. 2b). This was achieved by Red-mediated homologous recombination between a linear DNA and the endA gene. To do this, BW27783 cells were transformed with pBAD.Red. Cells from one transformed colony were used to make competent cells, as described[16]. Briefly, the cells were cultured in 25 ml of low salt LB containing ampicillin (50 μg/ml) and 1% L-arabinose and incubated at 32 °C with shaking at 250 r.p.m. until the OD600 was 0.5. The competent cells were immediately transformed with 50-ng of the Pme1-restricted targeting DNA fragment prepared from pK_anR.endA, cultured at 32 °C for 1 h, spread onto a plate containing kanamycin (Kan, 25 μg/ml) and incubated at 43 °C overnight. To select the KanR$^+$ colonies free of pBAD.Red, eight KanR$^+$ colonies were cultured in 500 μl LB with Kan at 43 °C for 30 min and 1 μl each was loaded onto the antibiotic-free, Kan$^+$, and Amp$^+$ plates, which were incubated at 43 °C overnight. To remove the KanR gene in the strain BWΔendA. KanR, competent cells were prepared from one KanR$^+$-colony, transformed with p2ΦC31 and spread onto a Amp$^+$ plate; subsequently, eight cultures were begun from eight p2ΦC31-transformed colonies in 500-μl antibiotic-free LB containing 1% L-arabinose at 32 °C for 2 h to induce the removal of KanR via ΦC31 integrase-mediated recombination between the attB and attP flanking the KanR, followed by 4 additional hours at 37 °C to induce I-SceI–mediated destruction of p2ΦC31. The cells were spread onto an antibiotic-free plate. Cells from eight colonies were cultured in 500 μl antibiotic-free LB at 37 °C for 30 min, and 1 μl each was loaded onto the antibiotic-free, Kan$^+$ and Amp$^+$ plates to select the AmpR$^-$/KanR$^-$ colonies. This resulted in the BWΔendA strain. The disruption of the endA gene was confirmed by DNA sequencing of the locus-specific PCR products generated from selected colonies.

Integration of the 2nd L-arabinose transporter lacY A177C and three copies of the BAD.I-SceI gene (Supplementary Fig. 2). Replacement of the *y* gene (lacY) of the lactose operon with the mutant lacY A177C (**Supplementary Fig. 2**) and integration of three copies of the I-SceI gene into the UMU locus (**Supplementary Fig. 3**) were achieved by the same Red-mediated homology recombination protocol as described for the endA gene disruption (**Fig. 2b**). To integrate the lacY A177C, however, knockout of the wild-type lacY was performed before knocking in the lacY A177C. Both were accomplished using the same Red-mediated homologous recombination. We used a DNA fragment containing TetR flanked with fragments of the *z* and *a* genes to knock out lacY, and selected the intermediate, BWΔendA. TetR on the Tet$^+$ (6 μg/ml)-plate. This allowed the selection of the next integrant using differential KanR/TetR selection (**Supplementary Figs. 2a**, **5a** and **6a**).

Integration of the BAD.ΦC31 cassettes. Three experiments were conducted to integrate ten copies of BAD.ΦC31 into the bacterial genome: two, four and four copies into the ΔendA locus (**Supplementary Fig. 4**), araD gene (**Supplementary Fig. 5**) and galK gene (**Supplementary Fig. 6**), respectively. All three integration events were achieved by ΦC31 integrase-mediated, site-specific recombination between the attB or attP site in an integrating plasmid, and the attB or attP in the targeted genomic sites as described for the endA gene disruption (**Fig. 2b**).

In an earlier attempt, we found that the integrated BAD.ΦC31 was lost soon after its integration. This is probably the result of a reverse reaction mediated by an uncharacterized excisionase or cofactor that worked in concert with ΦC31 integrase produced by the leaky expression from the integrated BAD.ΦC31. To stabilize the integrant, we designed a double recombination strategy. The ΦC31 integrase was used to mediate an integration event followed by FLP- or TPin-mediated recombination to remove the attL. This would eliminate the possibility of a reverse reaction between the attL and attR. For integration of the 2BAD.ΦC31 (**Supplementary Fig. 4**), we used the Red-mediated homologous recombination strategy with a linear DNA containing an attB site, a KanR gene and two flanking FRTs for insertion into the ΔendA locus. To remove the KanR, cells from one 3S2T.KanR colony were transformed with pcI587.FLP, and cells from a transformed colony were grown in 5 ml of antibiotic-free LB at 42 °C for 8 h. This induced the expression of FLP to mediate the recombination between the two FRTs, resulting in the elimination of KanR followed by the loss of the temperature-sensitive pcI587.FLP. Subsequently, the cells named 3S2T.attB were transformed with p2ΦC31.attP.FRT. Cells from one transformed colony were grown in 2-ml of LB containing 1% L-arabinose at 32 °C for 2 h, which induced the expression of ΦC31 integrase to mediate the recombination between the attB in the ΔendA locus and the attP in the plasmid. The same FLP-mediated FRT-FRT recombination was conducted to mediate the removal of the attL and R6K.Zeo and KanR, resulting in the strain of 2P3S2T (**Supplementary Fig. 4b**). The integrated 2BAD.ΦC31 was confirmed by DNA sequencing of the locus-specific PCR product (**Supplementary Fig. 4c**)

doi:10.1038/nbt.1708

and by demonstrating the function in mediating minicircle formation (**Supplementary Fig. 4e**). Integration of the four copies of the BAD.ΦC31 gene into the araD (**Supplementary Fig. 5**) and galK (**Supplementary Fig. 6**) loci, respectively, was also mediated by ΦC31 integrase; however, the removal of the attL was mediated by bacteriophage TP901-1 integrase (TPin). To do this, we used the Red recombination system to integrate a DNA fragment encoding TetR flanked with an attB and a phage attachment site of TPin (9attP) into the araD or galK site and confirmed the integrant by DNA sequencing of the integrant-specific PCR product (**Supplementary Figs. 5b** and **6b**). We integrated p4ΦC31.attP.9attB into the modified araD or galK locus following the same procedure as used for integrating the p2ΦC31.attP.FRT (**Supplementary Fig. 4**) with modifications. We selected the colonies with integrants using plates containing both Tet and Kan, transformed the selected intermediates 6P3S2T.KanR.TetR or 10P3S2T.KanR.TetR with plasmid pBAD.TPin, and screened the resulting colonies using triple antibiotic resistance (Tet, Kan and Amp). We grew the cells from one colony in 2 ml of antibiotic-free LB containing 1% L-arabinose, with shaking (250 r.p.m.) at 43 °C for 6–8 h before spreading onto an antibiotic-free plate and incubated at 43 °C overnight. L-arabinose induced expression of both recombinases, however, the incubation temperature allowed significant TPin integrase activity[18] but little ΦC31 integrase activity, resulting in the TPin integrase-mediated removal of attL and selection of colonies with the desired integrant, 6P3S2T (**Supplementary Fig. 5a**,**d**) and ZYCY10P3S2T (**Supplementary Fig. 6a**,**c**).

Minicircle production protocol. Our present minicircle producing system, the strain ZYCY10P3S2T plus the minicircle producer plasmid pMC.hFIX or pMC.RSV.hAAT, allowed for a greatly simplified minicircle production protocol (**Fig. 1b**). On day one, we inoculated cells from one transformed colony in 5 ml of TB (pH 7.0) with Kan (50 μg/ml) and incubated at 37 °C with shaking at 250 r.p.m. Later that day, we amplified the bacteria by combining 100 μl of culture to every 400 ml TB containing Kan (50 μg/ml) and continued incubation for 16 to 18 h. For the yield comparison study (**Fig. 2e**), a 400-ml overnight culture was used to prepare intact plasmid DNA. At the end of the culture period the A_{600} was 3.5– 4.2 with a pH of ~6.5. On day 2, we prepared a minicircle induction mix comprising 400 ml fresh LB, 16 ml 1N sodium hydroxide and 0.4 ml 20% L-arabinose, and combined it with a 400 ml overnight culture, and incubated the culture at 32 °C with shaking at 250 r.p.m. for an additional 5 h. We used Qiagen plasmid purification kits to isolate minicircle from bacterial lysates following the manufacturer's protocol with modifications. For every 400-ml overnight culture, we used a 2,500 column and 100-ml each of buffers P1, P2 and P3 to ensure complete resuspension and lysis of the bacteria and a high yield of minicircle DNA vector. A step-by-step protocol is provided in **Supplementary Protocol**.

qPCR determination of impurity DNAs. The presence of impurity DNAs, the unrecombined minicircle producer plasmid and plasmid backbone circle, in the three minicircle preparations was determined as described in the legends of **Figures 2d**,**e** by qPCR. To optimize DNA-polymerase activity, all the DNA templates were digested with AflII plus XhoI flanking the ColE1 origin before qPCR. The minicircle producer plasmid (**Supplementary Fig. 7**) without a transgene expression cassette was used as a standard. The PCR primers, 5′-TCCTGTTACCAGTGGCTGCT and 5′-AGTTCGGTGTAGGTCGTTCG, were specific for ColE1 DNA origin shared by all templates. qPCR was performed using the Qiagen Quant SYBR Green RT-PCR kit with a RCorbett system (Corbett). The program included a denaturing step of 94 °C for 10 min, followed by 25 cycles at 94 °C for 20 s, 55 °C for 15 s, and 68 °C for 20 s each.

16. Datsenko, K.A. & Wanner, B.L. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645 (2000).
17. Cherepanov, P.P. & Wackernagel, W. *Gene* **158**, 9–14 (1995).
18. Stoll, S.M., Ginsburg, D.S. & Calos, M.P. *J. Bacteriol.* **184**, 3657–3663 (2002).