Materials and Methods

Delimitation of the replication region of plasmid pANL

The origin of replication of pANL was mapped to a 10,832 bp *Bam*HI fragment (coordinates 36425-890 in GenBank Acc. No. AF441790) by (1). Further studies found that two non-overlapping *Kpn*I fragments of such *Bam*HI region, one comprising genes *sprE-sprL* and the other one including genes *anL55.5-anL59*, were both capable to sustain autonomous replication in Se7942 (2). The electrophoretic pattern of plasmid DNA samples rescued from Se7942 cells transformed with either of both replicons indicated that there were two classes of plasmids. A minority of samples presented restriction patterns identical to the plasmid used in the initial transformation of Se7942 cells. A second, more abundant class, corresponded to recombinants between the transformed plasmid and the endogenous pANL. In the case of the construction containing the complete *Bam*HI fragment, the proportion of autonomous plasmids recovered after transformation to Se7942 was significantly higher.

A cosmid-based deletion analysis suggested that there was only one replication origin in pANL (coordinates 42913-44307), which included CDS *anL55.5*, *anL56* and *anL56.5* (3). BLASTP hits with identity lower than 35% to these CDS are present in other cyanobacterial plasmids, where only *anL56* and *anL56.5* keep the synteny present in pANL. Such deletion analysis and a transposon-mediated mutagenesis approach also pointed to other plasmid regions as important for stable maintenance: i) CDS *anL29.5-anL33*, ii) *gap2* that includes coordinates 33455-33890, and iii) *gap3* (coordinates 35060-35811). Neither *gap2* nor *gap3* encoded a complete CDS. In this work, when using CDS *anL55.5-anL57* in the shuttle vector,

only cointegrates with pANL were rescued from Se7942 transconjugants. Nevertheless, the inclusion of antitoxin genes *anL29.5* and *anL33* clearly displaced the equilibrium to the obtaining of autonomously replicating units of the vector but depending on the culture conditions. Direct subculture of the Se7942 transconjugants in liquid BG11+Neo5, as performed for pDEP21 transconjugants, instead of replica plating on solid surface prior to subculture, as performed for pDEP23 transconjugants, could have somehow favored the non-recombinant form of the vector. Vector pDEP21 thus contains the smallest pANL replicon described so far that sustains autonomous replication.

Construction of the shuttle vectors

The origins of transfer (*oriT*) of conjugative plasmids RP4, pKM101, R388, R64 and F were amplified by PCR (30 cycles, 94ºC 30", 55ºC 30'' and 72ºC 30'') using primer pairs 1-2, 3-4, 5-6, 7-8 and 9-10, respectively. These primers contain 5' tails that harbor either *Eco*RI+*Xba*I or *Spe*I+*Pst*I restriction sites. Following the BioBrick cloning strategy (http://parts.igem.org/Help:Standards/Assembly), amplicons were digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, respectively obtaining plasmids pDEP11 to pDEP15.

The putative origin of replication of pANL (*rep*_pANL) (coordinates 42913 to 44327 of GenBank Accession No. AF441790) was amplified by PCR in two steps. First, a PCR using oligonucleotides 11 and 12 was carried out to obtain a 350 bp product lacking a *Pst*I site originally present in the template DNA (see Figure 1A) (30 cycles, 94ºC 30'', 55ºC 30'', and 72ºC 30''). Then, the resulting amplicon and oligonucleotide 13 were used as primers in a subsequent PCR (30 cycles 94ºC 30'', 55ºC 30'' and 72ºC 90'') to obtain *rep*_pANL. This PCR

fragment was digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, previously linearized with the same restriction enzymes, resulting in plasmid pDEP5.

Plasmid pDEP5 was digested with *Eco*RI/*Spe*I to obtain the fragment *rep*_pANL, which was cloned in the *Eco*RI/*Xba*I sites of pDEP11-pDE15 plasmids, producing pDEP6-pED10, respectively.

Steps to construct dislodging vectors pDEP21 and pDEP23

The *sepA1* antitoxin gene (coordinates 21506 to 21721 of GenBank Accession No. AF441790) was amplified by PCR in two steps. First, a PCR using primers 17 and 18 was carried out to obtain a 150 bp product lacking an *Xba*I site originally present in the template DNA (25 cycles, 94ºC 60'', 60ºC 30'', 72ºC 30''). The resulting amplicon and oligonucleotide 16 were used as primers in a subsequent PCR to obtain *sepA1* antitoxin gene (25 cycles, 94ºC 58'', 60ºC 30'', 72ºC 30''). The PCR fragment was digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, resulting in plasmid pDEP16. The *sepA2* antitoxin gene (coordinates 23754-24030, AF441790) was amplified by PCR (25 cycles, 94ºC 60'', 55ºC 30'', 72ºC 30'') using primers 19 and 20. The amplicon was digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, obtaining plasmid pDEP17. Promoter *Ptac* was obtained by hybridizing oligonucleotides 14 and 15 previously phosphorylated at their 5' ends. The hybridization product was digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, rendering plasmid pDEP18. Plasmid pDEP16 was digested with *Eco*RI/*Spe*I to obtain fragment *sepA1*, which was then cloned in *Eco*RI/*Xba*I sites of pDEP17, producing plasmid pDEP19 (pSB1K3::(*sepA1-sepA2*)). Plasmid pDEP18 was *Eco*RI/*Spe*I digested to obtain fragment *Ptac*, which was cloned in the *Eco*RI/*Xba*I sites of pDEP19, producing plasmid pDEP20 (pSB1K3::(*Ptac-sepA1-sepA2*)).

Finally, pDEP20 was restricted with *Eco*RI/*Spe*I to obtain fragment *Ptac-sepA1-sepA2*, which was cloned in the *Eco*RI/*Xba*I sites of pDEP6, obtaining plasmid pDEP21 (pSB1K3::(*Ptac-sepA1-sepA2-rep*_pANL-*oriT*$_{RP4}$)). The pANL region containing gaps 2 and 3 described by (3) and the sequence between them (coordinates 32843 to 36036 of GenBank Accession No. AF441790) was amplified by PCR (25 cycles, 94ºC 60'', 55ºC 30'', 72ºC 3'30'') using primers 21 and 22. This amplicon was *Eco*RI/*Pst*I digested and cloned in vector pSB1K3, rendering plasmid pDEP22. This plasmid was digested with *Eco*RI/*Spe*I to obtain fragment *gap2-3*, which was cloned in the *Eco*RI/*Xba*I sites of pDEP21, producing pDEP23 plasmid (pSB1K3::(*gap2-3-Ptac-sepA1-sepA2-rep*_pANL-*oriT*$_{RP4}$)).

Steps to construct a dislodging system based on *rps12*. The sequences flanking the maintenance region of plasmid pANL (homology sequence 1 (HS1, coordinates 19892-21004, AF441790) and homology sequence 2 (HS2, coordinates 24488-25464, AF441790)) were individually amplified by PCR (25 cycles, 94ºC 60'', 55ºC 30'', 72ºC 70''), respectively using primers 23-24 and 25-26. The amplicons were digested with *Eco*RI/*Pst*I and cloned in vector pSB1K3, producing plasmids pDEP24 and pDEP25, respectively. CDS *cat* and its promoter were amplified by PCR (25 cycles, 94ºC 60'', 55ºC 30'', 72ºC 50'') from vector pSB1C3 using primers 27-28. The amplicon was *Eco*RI/*Pst*I digested and cloned in pSB1K3, resulting in plasmid pDEP26. *Synechocystis* sp. PCC 6803 gene *rps12* under the control of the *psbA1* gene promoter (coordinates 1902-2589 of plasmid pEXR91) was amplified by PCR (25 cycles, 94ºC 60'', 55ºC 30'', 72ºC 50'') using primers 29-30. The amplicon was digested with *Eco*RI/*Pst*I and cloned in pSB1K3, giving pDEP27. This plasmid was restricted with *Eco*RI/*Spe*I to obtain gene *rps12*, which was cloned in the *Eco*RI/*Xba*I sites of pDEP26,

obtaining pDEP28 (pSB1K3::(*rps12-cat*)). Plasmid pDEP28 was in turn restricted with *Eco*RI/*Spe*I to obtain fragment *rps12-cat*, which was then cloned in the *Eco*RI/*Xba*I sites of pDEP25, producing pDEP29 (pSB1K3::(*rps12-cat*-HS2)). Finally, pDEP24 was restricted with *Eco*RI/*Spe*I to obtain fragment HS1, which was cloned in the *Eco*RI/*Xba*I sites of pDEP29, producing pDEP30 (pSB1K3::(HS1-*rps12-cat*-HS2)).

Supplementary Table S1. Oligonucleotides used in this work

Number	Description	Sequence (5’ -> 3’) [a]
1	*oriT*_RP4 nicked strand	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GCCGGCCAGCCTCGCAGAGCAG**
2	*oriT*_RP4 complementary strand	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***CAGCGCTTTTCCGCTGCATAACCCTG**
3	*oriT*_pKM101 nicked strand	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GCGGTTATTATTGGCATTCAGTCCTCAC**
4	*oriT*_pKM101 complementary strand	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***GAAGTGCCGCCCTGATACTTTTGGG**
5	*oriT*_R388 nicked strand	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GTCGCCTAGTGCCATGTCCTCTCCCG**
6	*oriT*_R388 complementary strand	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***TGCAAGCCCGGTTTCCGTCCG**
7	*oriT*_R64 nicked strand	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GAACTGACTCCTGGCAGGCTGCG**
8	*oriT*_R64 complementary strand	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***GGCTCCTTACGGGGTGTCGGG**
9	*oriT*_F nicked strand	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GAATGCAAACAGGGACGCACCG**
10	*oriT*_F complementary strand	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***ACATCAGGCAGATGGCTAACATCCA**
11	*rep*$_{\text{pANL}}$ forward for megaprimer construction	**GCTGAAGTCCTGCAACGCTGGCGTGACAGTG**
12	*rep*$_{\text{pANL}}$ reverse for megaprimer construction	*GTTTCTTCCTGCAGCGGCCGCTACTAGTATTA***T*****CA*****CTCGTTAAAGATCTGAAGAGCA**
13	*rep*$_{\text{pANL}}$ forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GGGCGATCTGCCCGTTTATTTTCTGC**

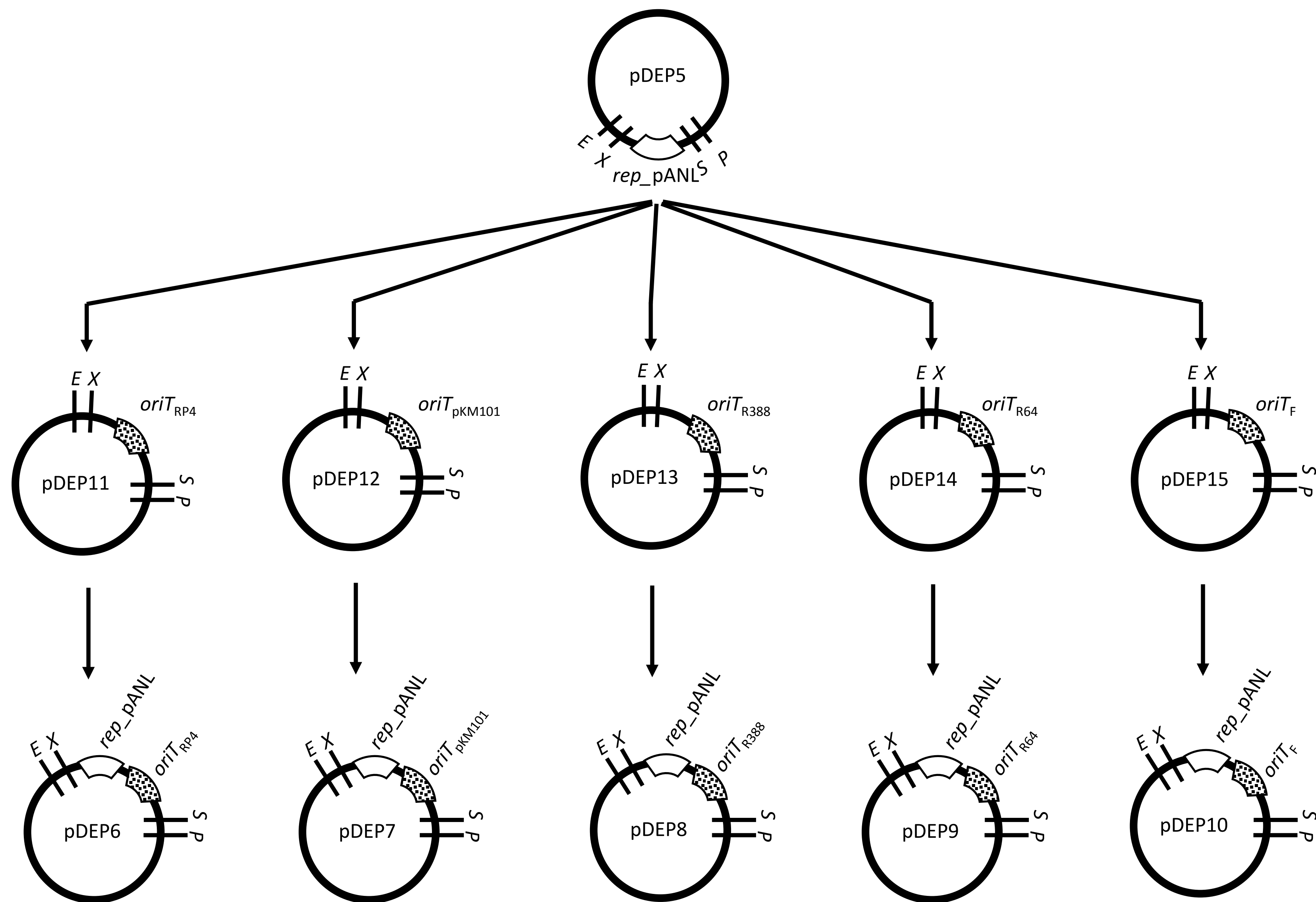
14	Ptac forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***TTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATT**
15	Ptac reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***AATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAAC**
16	sepA1 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***TTACTAAGGGCATGGAATGGGGGC**
17	sepA1 megaprimer forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GCTGATTTGGAGCTGAATTATGAATGC**
18	sepA1 megaprimer reverse	**GAGCTTTTCTTCCAGAACGGCCTGCTCTTC**
19	sepA2 forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GGAGCAGCTCCGATGGAAGCACATC**
20	sepA2 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***TTACTAGTCTTCAGGCCAGTCTTGACGG**
21	gap2-3 forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GTCCCTGAAGCCACAACTGCTG**
22	gap2-3 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***CCTTGGGATGACTCATGTTGGATATTG**
23	HS1 forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GGCCTGCGTAATGCGAGTCCAC**
24	HS1 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***CTGCTGGAGGATCACCGCAG**
25	HS2 forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***GGAGCGTATCAGCAGCCAGAGC**
26	HS2 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***GGCGGTCGATGTGCAAGC**
27	cat forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***CACGTAAGAGGTTCCAACTTTCACC**

28	cat reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***ATTACGCCCCGCCCTGCC**
29	PsbAI-rsp12 forward	*GTTTCTTCGAATTCGCGGCCGCTTCTAGA***TAGGGGGTTGCTCCTACGCTCAG**
30	PsbAI-rsp12 reverse	*GTTTCTTCCTGCAGCGGCCGCTACTAGTA***CACCTTCACTGGACAGGGGCG**
31	pANL-1 forward	**AGCCTTGTTGCCAGCATT**
32	pANL-1 reverse	**TTCCTGTTGTCGGGTCCG**
33	pANL-2 forward	**GGCAGGTCTTGTTTATTGAAGTG**
34	pANL-2 reverse	**CTGCTGAAGGTGAACCCG**
35	pANL-3 forward	**CGATGCACGATGTCGGTC**
36	pANL-3 reverse	**GATCGTGGGTCTATCAACACTG**
37	Maintenance region forward	**GCGTCTGGAGTCGAATCGTTGCC**
38	Maintenance region reverse	**TGATCGAACGTCTAGCCGCCG**
39	Test maintenance region presence forward	**GCGACGTTCGACAATTCTTCCCC**
40	Test maintenance region presence reverse	**GCCATTGGGATATATCAACGGTGG**

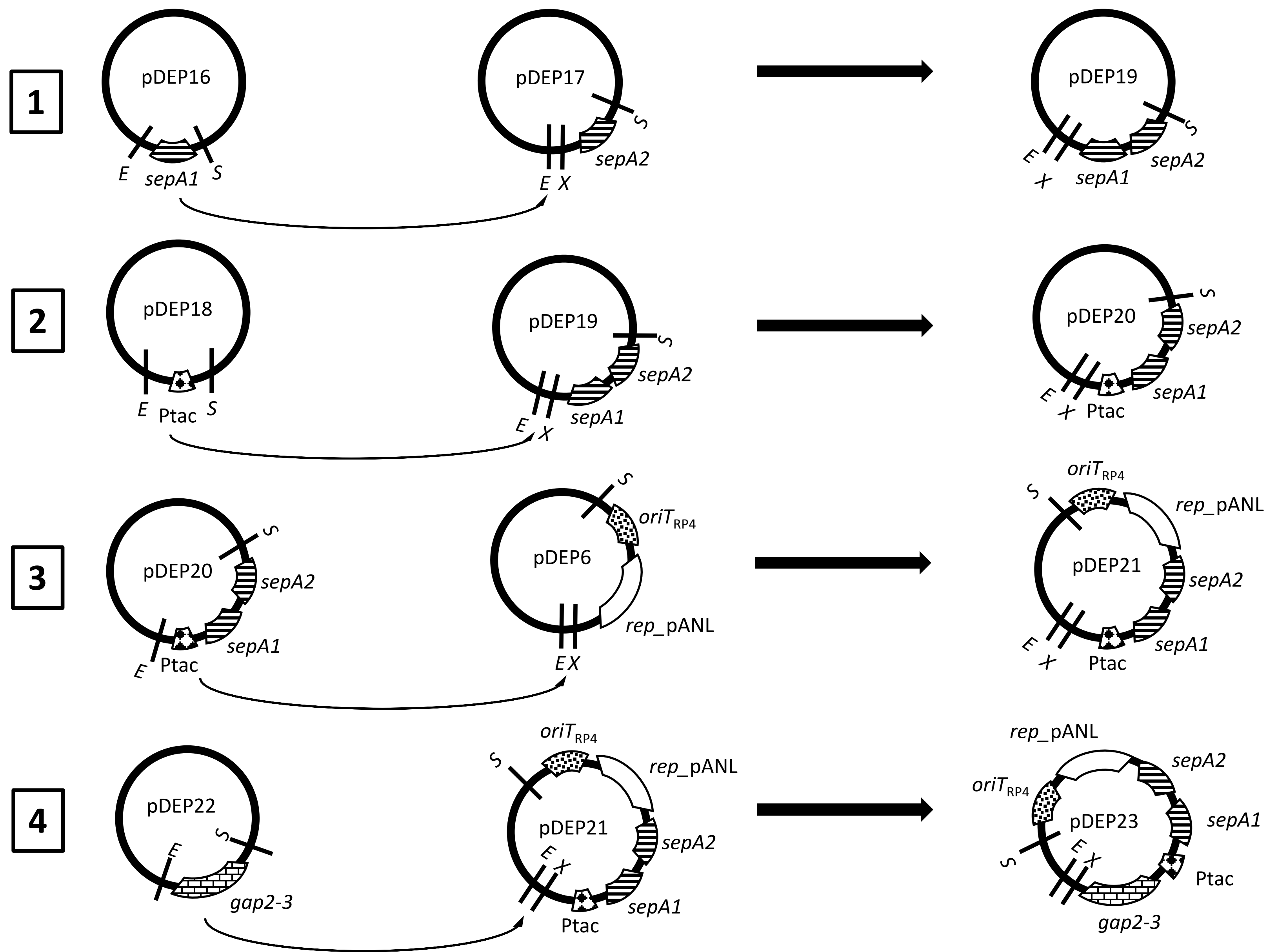
[a]: The BioBrick cloning tails are shown in italics and the restriction sites of the suffix or prefix underlined. In bold letters the sequences that hybridize with the template DNA, while mutant positions are indicated in bold underlined letters.

References:

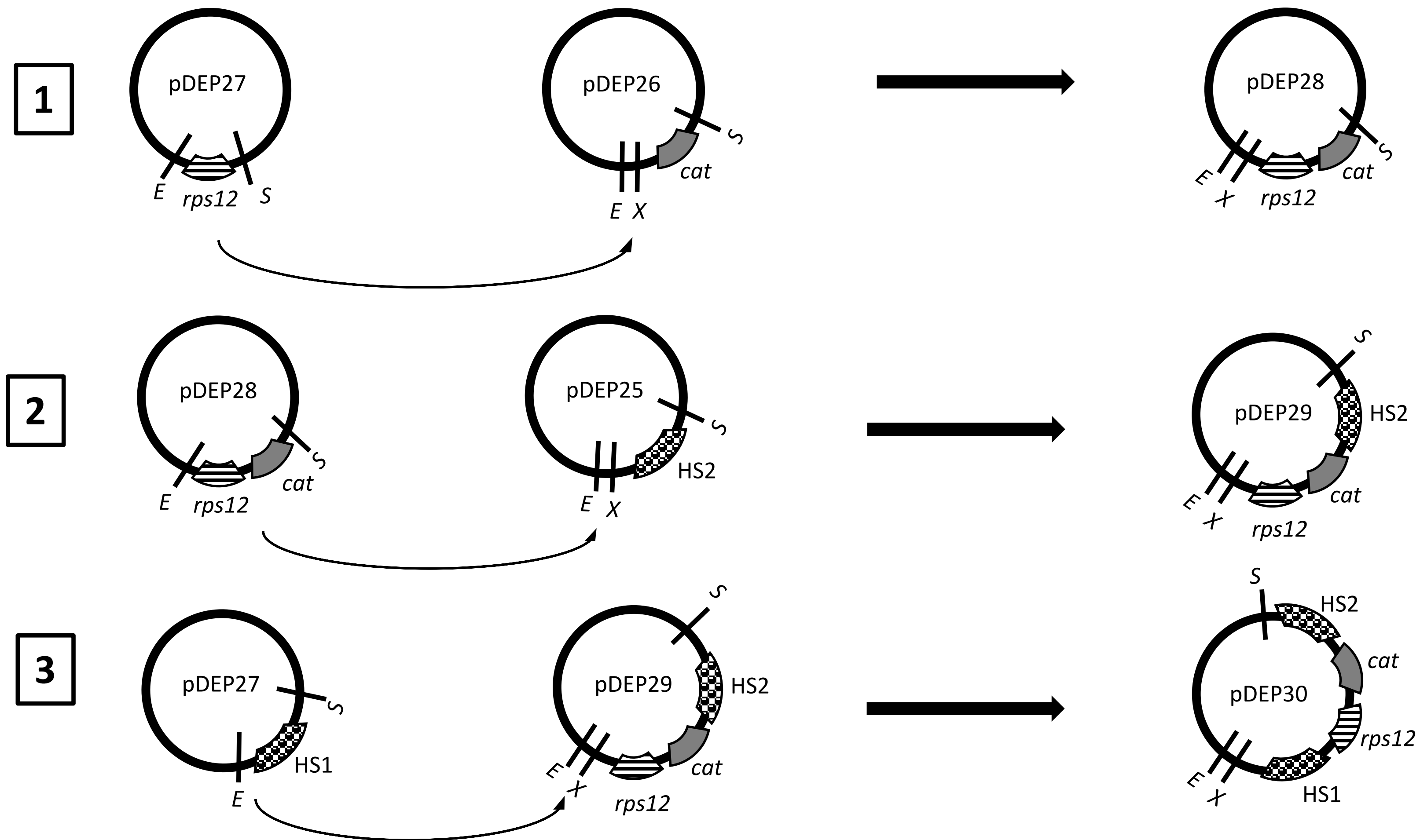
1. **Laudenbach DE, Strauss NA, Gendel S, Williams JP.** 1983. The large endogenous plasmid of Anacystis nidulans: mapping, cloning and localization of the origin of replication. Molecular & general genetics : MGG **192:**402-407.
2. **Laudenbach DE, Strauss NA, Williams JP.** 1985. Evidence for two distinct origins of replication in the large endogenous plasmid of Anacystis nidulans R2. Molecular & general genetics : MGG **199:**300-305.
3. **Chen Y, Holtman CK, Magnuson RD, Youderian PA, Golden SS.** 2008. The complete sequence and functional analysis of pANL, the large plasmid of the unicellular freshwater cyanobacterium Synechococcus elongatus PCC 7942. Plasmid **59:**176-192.



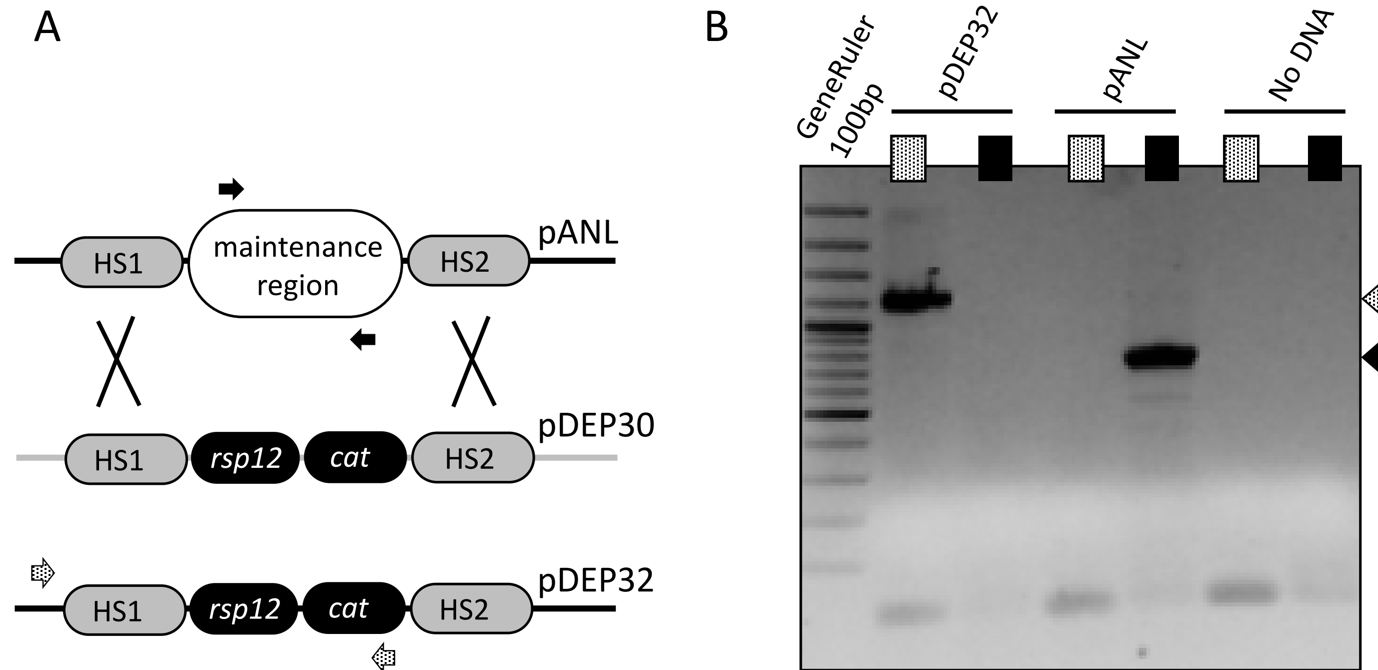
Supplementary Figure S1. Construction of the shuttle vectors. The origin of replication of Se7942 endogenous plasmid pANL (*rep*_pANL), as well as the origins of transfer (*oriT*) of conjugative plasmids RP4, pKM101, R388, R64 and F, were individually cloned as BioBrick parts in vector pSB1K3. The constructions were respectively named, pDEP5, pDEP11-pDEP15. These BioBrick parts are flanked by a prefix (*Eco*RI and *Xba*I restriction sites) and a suffix (*Spe*I and *Pst*I restriction sites). New parts can be sequentially added by restricting the vector with the prefix enzymes, while the parts by using *Eco*RI and *Spe*I. Vector-part *Eco*RI-*Eco*RI and *Xba*I-*Spe*I ligations guarantee the directionality of the insertion (the new part closer to the prefix), while restoring the prefix. Afterward, part *rep*_pANL was introduced in pDEP11-pDEP15 producing shuttle vectors pDEP5-pDEP10.



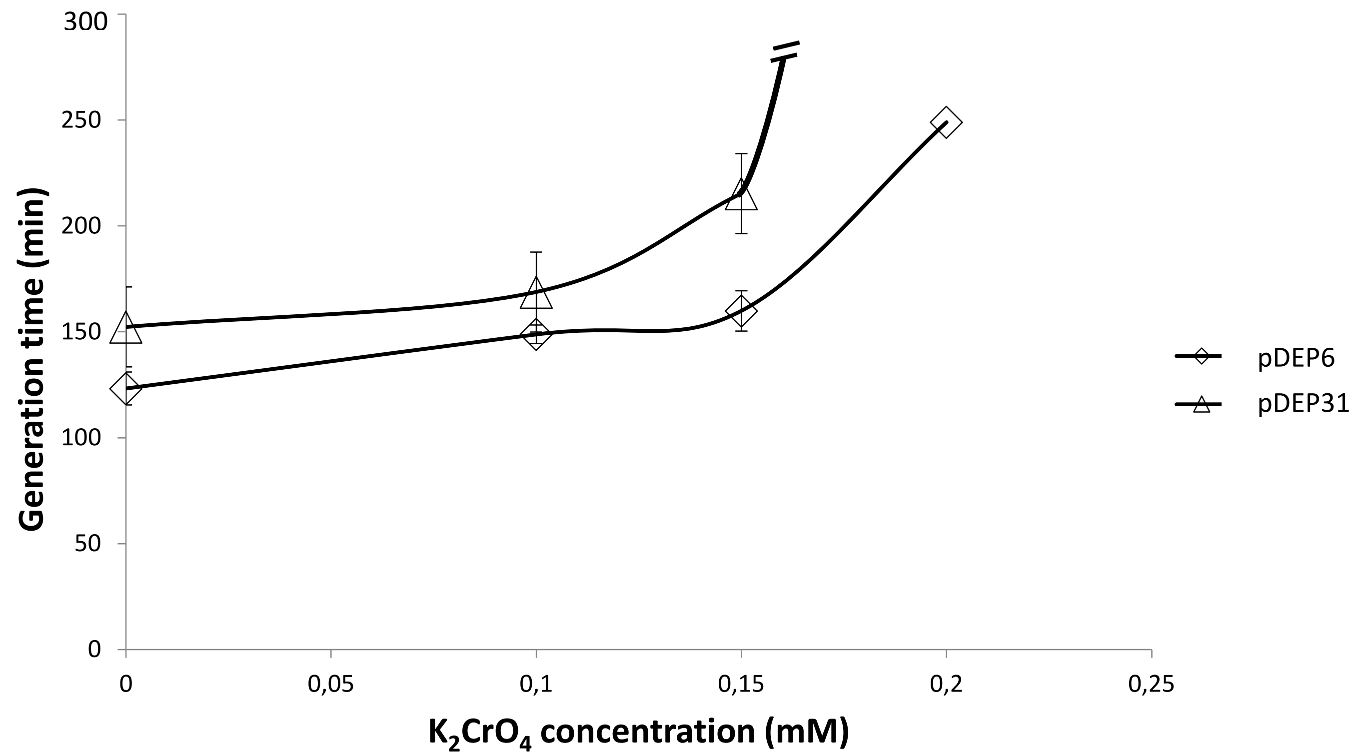
Supplementary Figure S2. Construction of the dislodging vectors. Following the BioBrick cloning strategy, antitoxin genes *sepA1* and *sepA2* were subsequently cloned (step 1) and put under the control of *Ptac* promoter (step 2). The set *Ptac-sepA1-sepA2* was transferred as a single BioBrick part to pDEP6, resulting in the first dislodging vector, pDEP21 (step 3). Inclusion of the pANL region comprising *gap2-3* in pDEP21 resulted in the second dislodging vector, pDEP23 (step 4).



Supplementary Figure S3. Construction of a dislodging system based on *rps12*. Genes *rps12* and gene *cat* were cloned (step 1). Then they were transferred as a single BioBrick part to a vector already containing a downstream flanking sequence of the maintenance region in pANL (HS2) (step 2). Finally, HS1, the upstream fragment flanking pANL maintenance region was incorporated, resulting in plasmid pDEP30 (step 3).



Supplementary Figure S4. pDEP32 analysis by PCR. (A) Deletion of the maintenance region of pANL by homology recombination with pDEP30 produced derivative pDEP32, which was checked by PCR. (B) Gel electrophoresis of the amplicons. Lanes indicated by a dotted box correspond to PCRs performed by using primers that hybridize upstream to HS1 and to *cat* gene (dotted arrows in panel A). An amplicon indicated by a dotted triangle is produced if the pANL maintenance region has been replaced by genes *rsp12* and *cat*. Lanes indicated by a black box correspond to PCRs performed by using primers that match pANL maintenance region (black arrows in panel B). No amplicon indicated by a black triangle is produced if recombination took place in all pANL copies.



Supplementary Figure S5: **Effect of the shuttle vector-pANL cointegrate in the growth of *E. coli*.** The doubling time of DH5α cultures with pDEP6 (open square) or the cointegrate pDEP31 (open triangle) was measured in the presence of increasing amounts of chromate. Each point is the average of 24 samples.