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Corresponding Author: Prof. Grazyna Jagura-Burdzy,

Corresponding Author's Institution: Institute of Biochemistry and Biophysics

First Author: Aneta Agnieszka Bartosik, Ph.D.

Order of Authors: Aneta Agnieszka Bartosik, Ph.D.; Aleksandra Markowska, M.Sc.; Jolanta Szarlak, M.Sc.; Anna Kulińska, Ph.D.; Grazyna Jagura-Burdzy

Abstract: Novel vectors for cloning and shuffling of gene cassettes based on minireplicon of broad-host-range RA3 plasmid from IncU incompatibility group were constructed. A series of minireplicon variants were prepared with copy number ranging from low (1-2 copies per chromosome), medium (10-15 copies per chromosome) to high copy number (80-90 copies per chromosome).
The new cloning vectors are relatively small in size (4.5-5.4 kb) and carry various resistance determinants: kanamycin (KmR), tetracycline (TcR) or chloramphenicol (CmR). The vectors were engineered to facilitate cloning and shuffling of the functional modules with or without transcriptional terminators. Using the described strategy, a bank of functional modules, ready for exchange, has been initiated.

To
Editor of Journal of Microbiological Methods

Dear Sir, Madam,

Please find attached the manuscript: "**Novel broad-host-range vehicles for cloning and shuffling of gene cassettes**" by Aneta A. Bartosik, Aleksandra Markowska, Jolanta Szarlak, Anna Kulińska and Grażyna Jagura-Burdzy. We present the novel broad-host-range vectors based on minireplicon of RA3 plasmid from IncU incompatibility group. Described plasmids were constructed for easy cloning and functional analysis of gene cassettes in the various bacterial species. They also offer the opportunity of gene cassettes shuffling to create "synthetic" plasmids. I am hoping you will consider the manuscript appropriate for publication in Journal of Microbiological Methods.

Yours sincerely

Grazyna Jagura-Burdzy

***Highlights**

Minireplicon of RA3 plasmid from IncU incompatibility group was exploited to create novel vectors for cloning and gene cassette shuffling in bacteria. New vectors are capable of replication in a broad range of hosts, are relatively small in size and carry various resistance determinants. The low, medium and high copy number vector variants were constructed. The vectors were engineered to facilitate cloning and shuffling of the functional modules with or without transcriptional terminators. Using described system gene cassettes may be easily linked together and exchanged creating novel plasmids with desired properties for various applications.

Novel broad-host-range vehicles for cloning and shuffling of gene cassettes.

Aneta A. Bartosik, Aleksandra Markowska, Jolanta Szarlak, Anna Kulińska and Grażyna Jagura-Burdzy*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences,
02-106 Warsaw, Pawinskiego 5A, Poland;

*Author for correspondence:
The Institute of Biochemistry and Biophysics, PAS, 02-106 Warsaw, Pawinskiego 5A, Poland,
Tel: +48 22 592 1212
Fax: +48 22 592 21 90
Email: gjburdzy@ibb.waw.pl

Running title: novel broad-host-range vectors

Keywords: IncU minireplicon; new biotechnology tools; library of functional modules; cassette shuffling.

Abstract

Novel vectors for cloning and shuffling of gene cassettes based on minireplicon of broad-host-range RA3 plasmid from IncU incompatibility group were constructed. A series of minireplicon variants were prepared with copy number ranging from low (1-2 copies per chromosome), medium (10-15 copies per chromosome) to high copy number (80-90 copies per chromosome).

The new cloning vectors are relatively small in size (4.5-5.4 kb) and carry various resistance determinants: kanamycin (Km^R), tetracycline (Tc^R) or chloramphenicol (Cm^R). The vectors were engineered to facilitate cloning and shuffling of the functional modules with or without transcriptional terminators. Using the described strategy, a bank of functional modules, ready for exchange, has been initiated.

1. Introduction

Sequencing of new bacterial genomes, metagenomic approaches and high-throughput experiments expand the available database enormously. For functional studies to complement the continuously growing sequence information as well as making the new information applicable there is a need to create novel vehicles to be used in a broad range of bacterial hosts. *E. coli* is a commonly used bacterial strain for genetic manipulations, such as gene cloning, protein over expression, construction of genome libraries, preparation of templates for sequencing, etc. Appreciation of the simplicity and safety of work with *E. coli* does not diminish the necessity to analyze the new gene functions or protein properties in their natural host. There are cloning vectors based on several broad-host-range replicons, e.g. RK2 of IncP-1 (Aakvik et al., 2009; Blatny et al., 1997; Crouzet et al., 1992; Ditta et al., 1985; Dombrecht et al., 2001; Kakirde et al., 2011; Santos et al., 2001; Scott et al., 2003; Thorsted et al., 1998), RSF1010 of IncQ (Bagdasarian et al., 1981; Chistoserdov and Tsygankov, 1986; Coppi et al., 2001; Frey, 1992; Gambill and Summers, 1985; O'Sullivan et al., 2010; Parales and Harwood, 1993; Priefer et al., 1985; Rangwala et al, 1991; Sharpe, 1984), pBBR1 of IncA/C (Kovach et al., 1994; Kovach et al., 1995) or rolling circle replicon originated from pWV01 (Bryksin and Matsumura, 2010). However, new systems compatible with those already available would be very useful.

Mobile genetic elements, among them bacterial plasmids with their loads, have a great potential of changing the properties of the host(s), and the challenge is to do it in a controllable way. Bioinformatic analysis of DNA sequences shows that many environmental plasmids have a precisely defined modular structure with blocks of genes devoted to the same plasmid function, e.g.: replication, conjugation or stable maintenance (Gerdes et al., 2000; Thomas, 2000; Toussaint and Merlin, 2002). Some of the plasmids have evolved highly complicated so called "global" regulatory networks to simultaneously control the expression of several modules or even coordinate the expression of the whole genome (Delver and Belogurov, 1997; Fernandez-Lopez et al., 2006; Macartney et al., 1997; Pansegrau et al., 1994; Zatyka et al., 1997). Others seem to rely on simple autoregulatory mechanisms (partition cassettes, TA-toxin-antidote operons) or autoregulatory mechanisms enhanced by additional regulatory circuits, but still encompassed within the boundaries of the functional module (replication cassettes) (Chattoraj et al., 1988; del Solar et al., 1998; Dunham et al., 2009; Friedman and Austin, 1988; Hirano et al.,

1998; Kulinska et al., 2011; Magnuson et al., 1996; Mukhopadhyay and Chattoraj, 2000; Ruiz-Echevarría et al., 1991). Growing knowledge of the interplay between the modules and understanding of their gene expression control make more realistic the perspective of linking functional modules of different origin into a new entity, so called "synthetic plasmid".

The aim of our studies was to construct a set of novel broad-host-range vectors of different copy number and resistance markers providing: (I) easy cloning procedure; (II) the possibility of cassette/gene function testing in various hosts ; (III) simple tools to create new synthetic plasmids by combining "bricks", the well-studied functional modules; (IV) easy exchange/shuffling of the modules.

Conjugative plasmid RA3 of IncU incompatibility group was originally isolated from *Aeromonas hydrophila*, but also repeatedly isolated from *E. coli* in hospital environments (Aoki et al., 1971; Rhodes et al., 2000; Tschape et al., 1981). The RA3 plasmid (45.9 kb) was shown to be stably maintained in *E. coli* and in the representatives of *Alpha-*, *Beta-* and *Gammaproteobacteria* (Kulinska *et al*., 2008). Minireplicon of RA3 plasmid was exploited to create novel vectors for cloning and gene shuffling in *E. coli*, capable of replication in a broad range of hosts and compatible with the already available BHR vectors.

2.Materials and methods

2.1. Bacterial strains and growth conditions.

Escherichia coli strain used was DH5α [F^-(*Φ80dlacZΔM15*) *recA1 endA1 gyrA96 thi-1 hsdR17*($r_k^- m_k^+$) *supE44 relA1 deoR Δ(lacZYA-argF)U196*] (Hanahan, 1983). *Pseudomonas putida* KT2442 rifampicin resistant strain (Rif^R) was provided by C.M. Thomas (University of Birmingham, UK). *Agrobacterium tumefaciens* LBA1010 strain (Rif^R) was provided by D. Bartosik (The Institute of Microbiology, University of Warsaw, Poland). Bacteria were generally grown in L broth (Kahn et al., 1979) at 37 or 30^{o}C. L agar (L broth with 1.5% w/v agar) was appropriately supplemented with antibiotics: benzyl penicillin, sodium salt 150 μg ml^{-1} in liquid media and 300 μg ml^{-1} in agar plates for penicillin resistance in *E. coli*; chloramphenicol 10 μg ml^{-1} in *E. coli*; kanamycin 50 μg ml^{-1} in *E. coli*, *P. putida* and *A. tumefaciens*; tetracycline 10 μg ml^{-1} and 30 μg ml^{-1} for tetracycline resistance in *E. coli* and in *P. putida*, respectively; rifampicin 50 μg ml^{-1} for all Rif^R strains. The L agar used for blue/white screening contained 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and Xgal at 40 μg ml^{-1}.

2.2. Plasmid DNA isolation, analysis, DNA amplification and manipulation.

Plasmid DNA manipulations were carried out by standard procedures (Sambrook et al., 1989). All plasmid constructs are listed in Table 1.

Standard PCR reactions (Mullis et al., 1986) were performed with appropriate pairs of primers listed in Table 2. All new plasmid constructs were sequenced. DNA sequencing was performed on a Pharmacia A.L.F. automatic sequencer (IBB, PAS, Warsaw) using Dye terminator kits supplied by the manufacturer. DNA sequence analysis was carried out using Clone Manager 9.

2.3. The read-through transcription test *in vivo*.

The plasmids pGBT70 and pGBT58 (Jagura-Burdzy et al., 1992) were modified to test the activity of the putative transcriptional terminator sequences. Both plasmids have *trfAp* of RK2 in the transcriptional fusion with promoter-less *xylE* encoding catechol 2,3-oxygenase. The pGBT70 carries the *trfAp-1* promoter-down mutation and exhibits 10-fold lower activity than WT *trfAp* inserted in the pGBT58. The 420 nt NcoI-KpnI fragment from between *trfAp* and *xylE* was replaced by annealed oligonucleotides #1 and #2 (Table 2) in both test plasmids. Insertion of the short 14 nt fragment eliminates NcoI cleavage site, but introduces additional single PstI and PscI recognition sites (type II restriction endonucleases NcoI and PscI recognize different sequences, but leave compatible sticky ends). This provides counter-selection after cloning of potential terminator sequence as KpnI-NcoI fragments into the vector digested with KpnI and PscI. The pairs of complementary oligonucleotides (Table 2) corresponding to the putative transcriptional terminator sequences were annealed and then cloned into the test plasmids pABB70.1 and pABB58.1.

To check the ability of putative transcriptional terminators to reduce transcription of *trfAp-xylE* fusion, the activity of catechol oxygenase was determined in logarithmically growing *E. coli* DH5α strains with the appropriate plasmids using a standard method (Zukowski et al., 1983). Protein concentration was assayed by the Bradford method (Bradford, 1976). One unit of 2,3 catechol oxygenase activity is defined as the amount of enzyme necessary to convert 1 μmol of substrate (catechol) to product in 1 min under standard conditions. The results were presented as % of read-through transcription compared to the control (100%) - a strain carrying the parental plasmid without inserted putative transcriptional terminator.

2.4. Site directed mutagenesis *in vitro*.

The mutagenic primers used in PCR reactions (Table 2) were designed individually for each mutation to contain the desired change in the nucleic acid sequence located centrally in the primer. The PCR reaction was performed according to the following cycler program: 5 min of initial denaturation at 95^{o}C, followed by 16 cycles of denaturation at 95^{o}C for 30 s, annealing at 55^{o}C for 1 min, elongation at 68^{o}C for 2 min per 1 kb of template length, with the final elongation step at 68^{o}C for 20 min. The PCR mixture was then treated for 1-2 h with 10 U of DpnI restriction enzyme in order to eliminate template DNA and then used for transformation of *E. coli* DH5α cells.

2.5. Bacterial transformation, electroporation and conjugation.

Competent cells of *E. coli* were prepared by standard $CaCl_2$ method (Sambrook et al., 1989). *P. putida* cells suitable for electroporation were prepared from liquid culture grown at 30^{o}C to OD_{600}=0.5 and washing the cells three times with ice cold deionized water and resuspending them at a high cell density (~ 1 x 10^{11} cells/ml) in ice cold 10% glycerol. The cells were dispensed in 40 µl aliquots into sterile microcentrifuge tubes and stored at -80^{o}C prior to electroporation. Ultrapure plasmid DNA (approximately 1µg) was added to the cells aliquot after thawing on ice. The mixture was placed into a 0.2 cm electroporation cuvette (Bio-Rad, Richmond, California.), and subjected to one pulse at 2.5 kV, 25 µF and 200 Ω using a Bio-Rad Gene Pulser. Sterile SOC solution (1 ml) was added and the contents transferred into a sterile microcentrifuge tube, incubated at 30^{o}C with shaking at 200 rpm for 1 h, then plated on L agar containing the appropriate antibiotic and grown at 30^{o}C overnight.

E. coli DH5α harboring pJSB15.9 (a pABB22 derivative with cloned $oriT_{RA3}$ region) or pJSB13.9 (a pABB20 derivative with cloned $oriT_{RA3}$ region) and either *E. coli* DH5α (pJSB1.24) with *tra* region of RA3 plasmid cloned into the pBGS18 vector or *E. coli* DH5α (RA3) were used as donor strains in triparental mating with chosen Rif^{R} recipient strains. Two donor strains and a recipient strain were mixed in a ratio 1:1:2 and 100 µl of the mixture was plated onto a non-selective L agar plates. After overnight incubation at 30^{o}C or 37^{o}C the bacteria were washed off the plate and dilutions were plated on appropriate selective media.

2.6. Plasmid stability test.

Cultures of bacterial strains with tested plasmids were grown in L broth containing the appropriate antibiotic at 30^{o}C or 37^{o}C. Plasmid content was checked by plasmid DNA isolation and agarose gel electrophoresis at the beginning of the experiment. The cultures were diluted

10^5-fold into fresh medium and grown without selection. Serial dilutions of the initial cultures were plated on L-agar and then a hundred colonies were transferred onto medium with antibiotic to estimate the percentage of antibiotic resistant clones. Approximately after 20 generations the cultures were diluted into fresh medium and samples of diluted cultures were plated onto L agar without antibiotic and then tested for antibiotic resistance. The stability of plasmid was assessed by the percentage of antibiotic resistant colonies at the beginning of the experiment and after 20 and 40 generations of growth without selective pressure.

2.7. Copy number.

The copy number of miniRA3 derivatives was estimated by Real-Time qPCR in the Laboratory for Genetic Modification Analyses of IBB PAS. Amplification and detection were carried out on an Applied Biosystems 7500 apparatus. Total bacterial DNA was purified with Genomic Mini purification kit (A&A Biotechnology) and treated with the restriction enzyme to linearize plasmid DNA. The *galK*, a single-copy gene on the chromosome of *E. coli*, was used as a reference gene (primers #23 and #24 Table 2), whereas the *repA* gene of RA3 was used as a target to estimate the copy number of the plasmid vector (primers #25 and #26 Table 2).

3. Results and discussion

3.1. The read-through transcription test *in vivo* to find effective transcription terminator signals.

Two terminator probe vectors with weak and strong promoters were used in the read-through transcription test *in vivo* (Fig. 1A). pGBT58 with *trfAp-xylE* transcriptional fusion carrying a strong promoter of *trfA* gene from the RK2 plasmid (IncP-1α) and pGBT70 with *trfAp*-xylE* fusion [weak *trfAp* with the promoter down mutation in -10 sequence, which decreased promoter activity 10-fold (Jagura-Burdzy et al., 1992)] were modified into pABB58.1 and pABB70.1, respectively, as described in section 2.3. The vectors were used to clone putative transcriptional terminators (annealed oligonucleotides listed in Table 2) between the promoter (*trfAp*) and the reporter gene (*xylE*). The use of the promoters with high and moderate transcriptional activity was rationalized to identify Rho-independent transcription terminators of high and low efficiency. The functionality of terminators was assessed by reduction of the transcriptional read-through from the promoter sequence leading to a decrease in the catechol oxygenase activity encoded by *xylE*.

Four Rho-independent transcriptional terminators, predicted *in silico* in P1 prophage (T_{pro}/ T_{lyz}; T_{aspmgT}/ T_{pap}) and RA3 plasmid (T_{nic}/T_{traC3}; T_{tnp513}) sequences (Łobocka et al., 2004; Kulinska et al., 2008), were cloned into the terminator probe vectors. Since three of those terminator sequences were presumed to function bidirectionally, they were cloned in both orientations in the test vectors. Six out of the seven tested transcriptional termination signals were functional in the applied test with *xylE* reporter gene under a moderate as well as a strong promoter (Fig. 1B). The T_{pro}/T_{lyz} P1 and T_{nic}/T_{traC3} RA3 were confirmed to be effective bidirectional terminators whereas the putative bidirectional sequence T_{aspmgT}/ T_{pap} of P1 showed termination only in one direction for T_{aspmgT}. Of the analyzed terminators the most potent was T_{tnp513} of RA3 leading to almost 100% reduction of XylE activity in both test vectors in comparison to the control. The T_{tnp513} and T_{nic}/T_{traC3} of RA3 as well as T_{pro}/T_{lyz} of P1 were chosen for use in the construction of plasmid vectors described in the next section.

3.2. Construction of pABB19 with T_{pro}/T_{lyz} P1 transcriptional terminator for gene cassette cloning.

To make a system of cassette banking universal and applicable for future shuffling of the desired fragments, it was decided to use (and recommended to other users) the defined pairs of restriction sites for cloning the functional modules. The choice of SalI at one end (producing compatible sticky ends with XhoI) and PscI at another (producing compatible sticky ends with NcoI and PagI) was meant to provide the way for selection of vectors with cloned inserts against empty ones. To avoid reading-through between multiple cassettes linked in the same vector, it was vital to extend the cloned fragments with transcriptional terminators if they did not possess them on their own.

To facilitate cloning of gene cassettes and their further manipulations in *E. coli*, a narrow-host range, high-copy number ampicillin resistant vector, derivative of pUC19 (Yanisch-Perron et al., 1985), was constructed. The original pUC19 vector sequence was modified by site-directed mutagenesis to introduce BclI and NcoI restriction sites next to the unique PscI cleavage site of pUC19 downstream of MCS (multiple cloning site). The T_{pro}/T_{lyz} P1 bidirectional transcriptional terminator sequence was then cloned between NcoI and PscI sites with BclI digestion of the ligation mixture as the counter-selection against parental vector to construct the cloning vector designated pABB19 (Fig. 2A). The localization of terminator sequence in PscI-NcoI fragment secures the vector sequences from unwanted strong transcriptional activities coming from the cloned modules and facilitates excision of the insert for further recloning and gene shuffling with or without a terminator sequence. So to create the bank of the cassettes for easy exchange, it is recommended to clone a potential gene cassette into pABB19 as the SalI-PscI or BamHI-PscI fragments (providing these are the unique sites for the insert) and then excise it as SalI-NcoI or BamHI-NcoI fragments with added transcriptional terminator for further manipulations.

3.3. Construction of pABB20 vector with T_{nic}/T_{traC3} RA3 transcriptional terminator for gene cassette cloning.

Gene cassette cloning in a high-copy-number vector might be difficult or even impossible due to the toxicity of the products of the cloned genes or their inhibitory effects on growth when expressed at a higher level. To avoid the gene dosage effect a low-copy-number plasmid, compatible with ColE1 replicons, derivative of RA3 minireplicon was constructed. RA3 is a broad-host-range, low-copy number, conjugative plasmid from the IncU incompatibility group

able to replicate in *Alpha-*, *Beta-* and *Gammaproteobacteria* (Kulinska et al., 2008). RA3 has a very clearly defined modular structure, its minireplicon encompasses *repA* and *repB* genes and a series of direct repeats upstream (DR1) and downstream (DR2) of the *rep* genes. The first version of the RA3 minireplicon (pMOB1.3.2) was obtained by linking of SnaBI fragment of the RA3 plasmid with kanamycin resistance gene excised from pKRP11 by HincII endonuclease (Reece and Philips, 1995)), followed by PvuII digestion and self-ligation (Ochocka M. and Jagura-Burdzy G., unpublished).

Due to the presence of remnants of insertion sequences in the flanking regions of a kanamycin resistance cassette originating from pKRP11, it was decided to replace a Km^R cassette cloned into pMOB1.3.2 by its shortened version from pDIY-KM vector deprived of these insertion sequences (Dziewit et al., 2011). Before the transfer of modified Km^R cassette into pMOB1.3.2, the three recognition sequences for XhoI, NruI and PagI in the Km^R cassette in pDIY-KM had to be eliminated for the purpose of future cloning. The XhoI and NruI cleavage sites were modified without changing the open reading frame in the Km^R gene by PCR-site directed mutagenesis, and a PagI restriction site, which is present in the non-coding region of the Km^R cassette, was modified after PagI digestion of pABB702 and blunt ending of 5' overhangs using the Klenow fragment of DNA Polymerase I and ligation, to give finally pABB703.

Simultaneously, a sequence within the RA3 minireplicon (pMOB1.3.2) was also changed by site directed mutagenesis to eliminate two EcoRI restriction sites present in the original sequence of the RA3 plasmid without changing the *repA* and *repB* open reading frames encoded within the region. A version of miniRA3, without two EcoRI sites and with the Km^R gene from pABB703, designated pABB708, was used as a starting point in the construction of modified cloning vectors.

The new derivative pABB708 was PCR-mutagenized (primers #37 and #38 Table 2) to add ApaI restriction site in close vicinity of the unique PvuII cleavage site in the non-coding region, upstream of the direct repeats (DR1) of RA3 minireplicon sequence. The ApaI-PvuII sites in the new derivative pABB709 where further exploited to insert a multiple cloning site sequence (MCS). The MCS sequence was made from two complementary oligonucleotides #21 and #22 (Table 2) after annealing, projected to leave sticky and blunt ends compatible with the ApaI-PvuII cleavage sites, respectively, and containing sequences recognized by NruI, BamHI, SalI, EcoRI, PscI and NcoI endonucleases. The ApaI restriction site was not rebuild after MCS

cloning in the obtained pABB710 plasmid, providing an opportunity for counter-selection of empty vector with ApaI digestion. In the next step, the potent transcriptional terminator sequence T_{nic}/T_{traC3} of RA3, made from two complementary oligonucleotides #5 and #6 (Table 2) was inserted into the pABB710 vector after PscI-NcoI digestion resulting in pABB20 vector (Fig. 2B). The transcriptional terminator was inserted at the end of the MCS to avoid read-through transcription from promoter sequences of cloning gene cassette on replicon sequences. For the purpose of future cloning and shuffling, the potential gene cassette should be inserted preferentially into the vector using SalI-PscI or BamHI-PscI restriction sites with the opportunity to use the EcoRI cleavage site for counter-selection against the empty vector.

The final product designated pABB20 is 4507 bp in size, carries the kanamycin resistance gene, MCS with NruI, BamHI, SalI, EcoRI, PscI restriction sites and T_{nic}/T_{traC3} RA3 bidirectional transcription terminator sequence at the end of MCS, between PscI-NcoI cleavage sites. The last modification facilitates consequent excision of the cloned gene cassette with or without the transcriptional terminator sequence (Fig. 2B).

Both pABB19 and pABB20 vectors can be used to clone, analyze and store single gene cassettes and provide them with Rho-independent transcription termination signals (Fig. 2A, B). For use of the cassettes to create synthetic plasmids (chosen combination of functional modules), it is vital that cloned inserts will be deprived of the recognition sequences not only for SalI, PscI and NcoI but also for PagI, XhoI, BamHI and NruI and eventually NotI and HindIII.

3.4. Construction of pABB21 and pABB22 derivatives for gene cassettes shuffling.

Two novel plasmid vectors, derivatives of RA3 minireplicon, were constructed for the purpose of cloning and linking together different gene cassettes. The pABB709 Km^R vector with eliminated EcoRI restriction sites in the replicon sequence and inserted ApaI cleavage site served as the backbone.

Initially the modified Cm^R resistance cassette obtained after HindIII digestion of pABB705 replaced the Km^R cassette in pABB709 to produce plasmid pABB711.

In the next stage, the ApaI-PvuII restriction sites of pABB711 were used to insert the first part of the MCS sequence made from two annealed complementary oligonucleotides #17 and #18 (Table 2). The ApaI site was not reconstructed after cloning in resultant pABB712 vector to facilitate post ligation counter-selection against the empty vector. The potent, unidirectional

T_{tnp513} RA3 terminator sequence (Fig. 1B) was then inserted (as two annealed oligonucleotides #3 and #4 Table 2) at the end of MCS between the KpnI-PagI cleavage sites of pABB712. In the derivative plasmid pABB713 the PagI restriction site was not rebuilt after terminator sequence insertion. The NcoI cleavage site in the terminator sequence was eliminated by NcoI digestion and blunt ending of 5'overhangs using the Klenow fragment of DNA Polymerase I to produce plasmid pABB714. To obtain a full range of planned restriction sites in MCS, the second part of MCS was introduced into the vector on two annealed complementary oligonucleotides #19 and #20 (Table 2) with inactivation of the KpnI cleavage site after ligation. It resulted in plasmid pABB715 harboring the multiple-cloning site with EcoRV, XhoI, PscI, SalI, PagI, BamHI and NcoI cleavage sites, T_{tnp513} RA3 terminator sequence behind the NcoI site and one NotI restriction site between the transcription terminator sequence and the PvuII restriction site (Fig. 2C). A second NotI restriction site was introduced by PCR- site directed mutagenesis at the end of DR2 direct repeats of the RA3 minireplicon sequence utilizing pABB715 as a template and #41 and #42 primers (Table 2). The final product was designated pABB21 (Fig. 2C).

To be able to test the constructed plasmid using tetracycline resistance, the Cm^R resistance cassette in pABB21 was replaced by tetracycline (Tc^R) resistance gene from pKRP12 utilizing HindIII digestion. The resultant plasmid pABB16 was then used as a template in a PCR reaction with primers #43 and #44 (Table 2) to inactivate the EcoRV restriction site present in the Tc^R sequence and obtain plasmid pABB17. In the next step, the PagI cleavage site was also modified in the tetracycline resistance gene by site directed mutagenesis using the pABB17 plasmid as a template and #45 and #46 primers (Table 2). This resulted in the cloning vector plasmid pABB22 with a modified Tc^R cassette.

In the final step, all constructed cloning vectors were completely sequenced to confirm the fidelity of DNA sequences.

The constructed novel vectors, pABB21 and pABB22, carrying, chloramphenicol (Cm^R) and tetracycline (Tc^R) resistance genes, respectively, are relatively small of 4425 and 5432 bp, and contain a multiple cloning site facilitating orderly cloning of several independent gene cassettes without or with transcriptional terminators to prevent read-through transcription from internal promoters. At the end of the multiple cloning site in both vectors there is also a strong, unidirectional transcriptional terminator T_{tnp513} from RA3. The plasmids differ in the potential number of cloned cassettes since there are two extra restriction sites enabling cloning of blunt

ended fragments downstream of T_{tnp513} in pABB21 (Fig.2C, D and E). The presence of two NotI restriction sites, at the end of MCS and the end of replicon of pABB21 or pABB22, facilitates excision of the RA3 minireplicon to secure autonomy of the potentially cloned replication cassette. Additionally, there is also the possibility to exchange or excise the resistance gene cassette in pABB21 or pABB22 utilizing HindIII digestion.

3.5. Host range of miniRA3 derivatives.

The ability of constructed vectors to replicate in several bacterial strains belonging to different classes of *Proteobacteria* (*Alpha-* and *Gammaproteobacteria*) was analyzed. Plasmid DNA isolated from *E. coli* DH5α was used for transformation or electroporation of different bacterial host. Tested vectors were efficiently introduced either by transformation or electroporation only to competent cells of *E. coli* DH5α and *P. putida* KT2442 strains. Using three parental conjugation method pJSB15.9 (derivative of pABB22 with $oriT_{RA3}$) or pJSB13.9 (derivative of pABB20 with $oriT_{RA3}$) were mobilized by pJSB1.24 or RA3, respectively, to *E. coli* DH5α, *P. putida* KT2442 and *A. tumefaciens* LBA1010 strains. Plasmids relying on miniRA3 replicon (derivatives of pABB20 with cloned $oriT_{RK2}$) were introduced by conjugation [using *E. coli* S17-1 with appropriate plasmid as a donor strain (Simon et al., 1983)] and found to replicate in *Paracoccus aminovorans* JCM7685, *Paracoccus pantotrophus* KL100, *Paracoccus solventivorans* DSM11592, *Brevundimonas* sp. LM18R, *A. tumefaciens* LBA1010 (M. Szuplewska and D. Bartosik, personal communication). This confirmed the broad host range of the RA3 minireplicon and the usefulness of the constructed vectors in studies in various bacterial species.

3.6. Construction of new derivatives of vectors based on RA3 minireplicon with higher copy number.

The copy number of RA3 has been established using Real-Time quantitative PCR analysis (qPCR) as approximately one copy per chromosome in *E. coli* and *P. putida* (Kulinska et al., 2008). The RA3 minireplicon, pMOB1.3.2 demonstrates 2-3 copies per chromosome in *E. coli* DH5α. Studies on RA3 replication system showed that some mutations mapping in the regulatory elements of minireplicon may significantly increase the plasmid copy number, e.g. pAMB2.4 and pAMB2.5, Table 1 (Markowska A. and Jagura-Burdzy G., unpublished). The

same mutations were introduced by PCR site-directed mutagenesis into pABB21 with two pairs of primers #47 and #48 and #49 and #50 (Table 2) to construct pAMB21.4 and pAMB21.5, respectively. The copy number of pABB21 and its derivatives pAMB21.4 and pAMB21.5 was tested in *E. coli* DH5α strain. As visualized by agarose gel electrophoresis of plasmid DNA (Fig. 3A) and confirmed by qPCR, pABB21 is a low copy number vector (1-2 copies per chromosome). The pAMB21.4 vector was estimated to be a medium copy number plasmid (similarly to pAMB2.4) with approximately 15 copies per chromosome. The pABB21.5 demonstrated the highest plasmid copy number, approximately 90 copies per chromosome in *E. coli* DH5α strain (similarly to pAMB2.5). The pABB21 and its derivatives with elevated copy number might provide valuable tools for cloning and analysis of gene dosage effects in various hosts.

3.7. Stability of new vectors in different hosts.

The transformants of *E. coli* DH5α harboring miniRA3 derivatives were grown without selection for up to 40 generations and monitored for plasmid stability. All tested *E. coli* transformants retained the antibiotic resistance in 100% for 40 generations of growth without selection (Fig. 3B), demonstrating that all constructed miniRA3 derivatives are very stably maintained in this host.

Under selective conditions the tested *P. putida* KT2442 transformants of miniRA3 derivatives also retained the plasmids in 96-100% of cells (Fig. 3C); however, under non-selective conditions the tested plasmids were less stable than in *E. coli*. The pMOB1.3.2 exhibited about 80% retention after 20 generations of non-selective growth and 60% retention after 40 generations of non-selective growth. For pABB20 and pABB22 transformants after 20 generations of growth without selective pressure approximately 45% and 10% cells, respectively, possessed the plasmid. Interestingly, pABB22 derivative with inserted $oriT_{RA3}$ (pJSB15.9) was very stably maintained in *P. putida* (as well as in *E. coli* cells) and after 40 generations of growth without selection 100% colonies still exhibited resistance to antibiotic. This vector can be mobilized by RA3 conjugal transfer system when provided *in trans* and has been successfully used to introduce miniRA3 replicon into different bacterial hosts by conjugation (section 3.5).

The pAMB2.4 (a medium copy number derivative of pMOB1.3.2) when tested in *P. putida* showed similar retention as its parental, low copy number plasmid pMOB1.3.2 (Fig. 3C).

The pAMB2.5, other derivative of pMOB1.3.2 with high copy number, tested in *P. putida* exhibited even lower stability than pMOB1.3.2 (less than 40% of the cells contained the plasmid after 20 generations). This showed that an increase in the plasmid copy number is not directly related to the higher plasmid stability, at least in *P. putida* KT2442 strain.

The transconjugants of pABB20 derivatives in *P. solventivorans* DSM11592 and *A. tumefaciens* LBA1010 (kindly supplied by Szuplewska M. and Bartosik D.) were also analyzed for plasmid retention after growth under non-selective conditions. The pABB20 derivative was highly stable in *P. solventivorans* DSM11592 (approximately 90% plasmid retention after 20 generations of growth without selective pressure). The same derivative in *A. tumefaciens* LBA1010 was less stable demonstrating only 60% retention after 20 generations.

It is then highly recommended to check the stability of chosen derivative in the particular host strain since slight modification of plasmid size or selective marker used may influence the plasmid retention.

3.8. Gene cassette cloning and shuffling using the constructed vectors.

The described vectors were constructed for two main purposes: cloning, storage and functional analysis of modules on one hand and cassette shuffling on another.

Mainly, pABB19 (high-copy-number, narrow-host range) and pABB20 (low-copy-number, broad-host-range) might be useful in cloning and verification by sequencing and functional analysis of the inserted gene cassettes. A broad spectrum of gene cassettes cloned independently and verified in pABB19 or pABB20 may be accounted as the library of functional modules, potential collection of "bricks" from which new plasmids may be formed. For the purpose of further linkage of two or more gene cassettes, the single gene cassette should be inserted into pABB19 and pABB20 between SalI-PscI or BamHI-PscI cleavage sites with possible counter-selection of the parental vector in both cases using appropriate restriction endonucleases (Fig. 2A, B).

In the next step, selected gene cassettes can be linked together using one of the available versions of the vector for cloning and shuffling different gene cassettes - the pABB21 derivatives (Cm^R) or pABB22 (Tc^R). Both vectors can be used to link at least four independent gene cassettes whereas pABB21 may easily accommodate even six cassettes.

Figure 2E shows exemplary scheme of cloning of independent gene cassettes using constructed vectors pABB21 or pABB22. Sequential linking of few gene cassettes into pABB21 or pABB22 relies on the choice of specific restriction sites for cloning producing compatible sticky ends e.g. SalI/ XhoI, NcoI/ PscI/ PagI restriction sites as well as EcoRV, NruI, PvuII or NaeI restriction sites recognized by endonucleases producing blunt ends after cleavage and providing the means for selection of vectors with cloned inserts against the empty ones. For sticky ends cloning procedure its recommended to excise a potential gene cassette cloned in pABB19 or pABB20 as the SalI-PscI or BamHI-PscI without transcriptional terminator, or SalI-NcoI or BamHI-NcoI fragments with added transcriptional terminator, and insert it into pABB21 or pABB22 after XhoI-PscI, SalI-PagI or BamHI-NcoI digestion, respectively, choosing appropriate endonuclease for counter-selection of the "empty" vector. In this way three independent gene cassettes with or without added transcriptional terminator might be cloned in the chosen vector for shuffling of gene cassette. The EcoRV cleavage site of pABB21 and pABB22 and blunt ends ligation without reconstruction of the EcoRV restriction site might be exploited to clone a fourth gene cassette after its excision from pABB20 derivative using NruI-PvuII restriction sites. In this case, the gene cassette is excised from the vector together with the terminator sequence and can be cloned in two possible orientations (Fig. 2E).

pABB21 vector with Cm^R resistance gene can be used to link together as many as six independent gene cassettes exploiting the additional unique NaeI and PvuII cleavage sites. In pABB22, due to the presence of additional NaeI and PvuII restriction sites within the Tc^R cassette, these sites cannot be used to insert further gene cassettes. Accommodation of gene cassettes at positions I to IV provides opportunity to remove the original minireplicon of RA3 from pABB21 and pABB22 by using NotI digestion (Fig. 2C, D, E). This is the case when one of the cloned gene cassettes harbors, an autonomous replication system. It is worth mentioning that PvuII and NaeI restriction sites are present in pABB21 downstream of the NotI cleavage site belonging to MCS; hence NotI digestion cannot be used to cut out miniRA3 replicon from pABB21 in which NaeI, PvuII cleavage sites were exploited to insert extra gene cassettes. The HindIII digestion can be applied to change the resistance gene cassette in pABB21 or pABB22 vectors. It is important to remember that all of the above cloning procedures with the use of particular restriction enzymes might be utilized only if the cleavage sites planned to be used are not present (had been previously modified) in the gene cassettes of interest.

4. Conclusions

Novel cloning vectors have been prepared based on the broad-host-range minireplicon of RA3 plasmid from IncU incompatibility group. They can be used for cloning and analysis of the gene function, promoter activities and protein overproduction in different hosts outside of the *Enterobacteriaceae* family. These are low, moderate- and high-copy-number plasmids with different resistance markers for screening in the wide range of hosts. The easy-cloning strategy relies on the use of restriction enzymes with various specificities but producing compatible ends, so that a new insert would lead to the disappearance of the restriction site, and this will be utilized as a counter-selection to increase the frequency of insertion.

The main purpose of construction of these novel vehicles has been to prepare tools for combination of different functional modules into "synthetic" plasmids for particular biotechnological tasks.

A two-step strategy has been planned to clone the well-defined functional modules to create a universal bank of replication, stability, mobilization and conjugative transfer modules. It can be expanded by controllable expression systems, a wide spectrum of resistance markers, degradative or biosynthetic operons, etc. The first step is the cloning of the cassette into prepared high- or low-copy number vector to provide it, if required, with Rho-independent transcription termination sequences. At this stage the DNA sequence of the cassette should be known (or may be established) and modified according to the requirements (removal of the recognition sites for certain restriction endonucleases by PCR site-directed mutagenesis). The second step is combining the functional modules of choice into new plasmids. Using the strategy described above, up to six different modules may be linked. The transcription terminator sequences protect the "bricks" from transcriptional interference. In our system we offer also three variants of miniRA3 replicon for cassette shuffling: low-, medium- and high-copy number plasmids, and two variants of resistance markers inserted. The replication system of broad-host-range RA3 plasmid may be easily replaced by a replication cassette more appropriate for the analyzed host; the same applies to the resistance marker. The creation of a universal banking system for functional bacterial modules has a great potential and use of our system of cassettes shuffling is highly recommended.

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Table(s)

Table 1. **Plasmids used in this study.**

Plasmids provided by others:		
Plasmid name	**Relevant features**	**References**
pGBT58	*ori*$_{pSC101}$, Km^R, *trfAp*$_{RK2}$-*xylE*	Jagura-Burdzy et al., 1992
pGBT70	*ori*$_{pSC101}$, Km^R, *trfAp-1*$_{RK2}$-*xylE*	Jagura-Burdzy et al., 1992
pUC19	*ori*$_{MB1}$, Ap^R, cloning vector	Yanisch-Perron et al., 1985
pBGS19	*ori*$_{MB1}$, Km^R, cloning vector	Spratt et al., 1986
pKRP10	*ori*$_{MB1}$, Ap^R, Cm^R cassette	Reece and Phillips, 1995
pKRP11	*ori*$_{MB1}$, Ap^R, Km^R cassette	Reece and Phillips, 1995
pKRP12	*ori*$_{MB1}$, Ap^R, Tc^R cassette	Reece and Phillips, 1995
pDIY-KM	*ori*$_{MB1}$, Ap^R, modified Km^R cassette	Dziewit et al., 2011
pMOB1.3.2	miniRA3Km^R with inactivated EcoRI cleavage site within *repB* gene	Ochocka M. and Jagura-Burdzy G., unpublished
Plasmids constructed during this work:		
	Description, relevant features	
pABB16	pABB21 with Cm^R cassette replaced by Tc^R resistance cassette originating from pKRP12 using HindIII digestion	
pABB17	pABB16 with modified EcoRV restriction site within Tc^R cassette (PCR directed mutagenesis with primers #43 and #44)	
pABB19.1	pUC19 with added BclI, NcoI restriction sites downstream of existed PscI cleavage site (PCR directed mutagenesis with primers #39 and #40)	
pABB19	*ori*$_{MB1}$, Ap^R, transcriptional terminator T$_{pro}$/T$_{lyz}$ P1 (inserted between PscI- NcoI restriction sites of pABB19.1), cloning vector, 2732 bp in size	
pABB20	*ori*$_{RA3}$, Km^R, transcriptional terminator T$_{nic}$/T$_{traC3}$ RA3 (inserted between PscI-NcoI restriction sites of pABB710), cloning vector, 4507 bp in size	
pABB21	*ori*$_{RA3}$, Cm^R, transcriptional terminator T$_{tnp513}$ RA3, vector for gene cassettes linking and shuffling, 4425 bp in size, derivative of pABB715 with added NotI restriction site downstream of miniRA3 replicon (PCR directed mutagenesis with primers #41 and #42)	
pABB22	*ori*$_{RA3}$, Tc^R, transcriptional terminator T$_{tnp513}$ RA3, vector for gene cassettes linking and shuffling, derivative of pABB17 with PagI cleavage site modification within Tc^R cassette (PCR directed mutagenesis with primers #45 and #46)	
pABB58.1	pGBT58 derivative with 420nt NcoI-KpnI fragment from between *trfAp* and *xylE* replaced by short linker (annealed oligonucleotides #1 and #2*)* providing unique PstI and PscI recognition sites	
pABB70.1	pGBT70 derivative with 420nt NcoI-KpnI fragment from between *trfAp* and *xylE* replaced by short linker (annealed oligonucleotides #1 and #2*)* providing unique PstI and PscI recognition sites	
pABB58.2	pABB58.1 with inserted transcriptional terminator *T$_{tnp513}$* RA3 (annealed oligonucleotides #3 and #4*)*	
pABB58.3	pABB58.1 with inserted T$_{nic}$/T$_{traC3}$ RA3 terminator (annealed oligonucleotides #5 and #6*)*	
pABB58.4	pABB58.1 with inserted T$_{traC3}$/T$_{nic}$ RA3 terminator (annealed oligonucleotides #7 and #8*)*	
pABB58.5	pABB58.1 with inserted T$_{pap}$/T$_{aspmqT}$ P1 terminator (annealed oligonucleotides #9 and #10*)*	
pABB58.6	pABB58.1 with inserted T$_{aspmqT}$/ T$_{pap}$ P1 terminator (annealed oligonucleotides #11 and #12*)*	
pABB58.7	pABB58.1 with inserted T$_{pro}$/T$_{lyz}$ P1 terminator (annealed oligonucleotides #13 and #14*)*	
pABB58.8	pABB58.1 with inserted T$_{lyz}$/T$_{pro}$ P1 terminator (annealed oligonucleotides #15 and #16*)*	
pABB70.2	pABB70.1 with inserted transcriptional terminator *T$_{tnp513}$* RA3 (annealed oligonucleotides #3 and #4*)*	
pABB70.3	pABB70.1 with inserted T$_{nic}$/T$_{traC3}$ RA3 terminator (annealed oligonucleotides #5 and #6*)*	
pABB70.4	pABB70.1 with inserted T$_{traC3}$/T$_{nic}$ RA3 terminator (annealed oligonucleotides #7 and #8*)*	
pABB70.5	pABB70.1 with inserted T$_{pap}$/T$_{aspmqT}$ P1 terminator (annealed oligonucleotides #9 and #10*)*	
pABB70.6	pABB70.1 with inserted T$_{aspmqT}$/ T$_{pap}$ P1 terminator (annealed oligonucleotides #11 and #12*)*	
pABB70.7	pABB70.1 with inserted T$_{pro}$/T$_{lyz}$ P1 terminator (annealed oligonucleotides #13 and #14*)*	
pABB70.8	pABB70.1 with inserted T$_{lyz}$/T$_{pro}$ P1 terminator (annealed oligonucleotides #15 and #16*)*	
pABB701	pDIY-KM with inactivated XhoI cleavage site within Km^R cassette (PCR directed mutagenesis with primers #27 and #28)	
pABB702	pABB701 with inactivated NruI cleavage site within Km^R cassette (PCR directed mutagenesis with primers #29 and #30)	
pABB703	pABB702 with inactivated PagI cleavage site within Km^R cassette by blunting the ends and	

	ligation
pABB704	pKRP10 with inactivated NcoI cleavage site within Cm^R cassette (PCR directed mutagenesis with primers #31 and #32)
pABB705	pABB704 with inactivated PvuII cleavage site within Cm^R cassette (PCR directed mutagenesis with primers #33 and #34)
pABB706	pMOB1.3.2 with inserted Cm^R instead of Km^R using HindIII digestion
pABB707	pABB706 with inactivated EcoRI cleavage site within *repA* gene (PCR directed mutagenesis with primers #35 and #36)
pABB708	pABB707 with Cm^R cassette replaced by modified Km^R cassette originating from pABB703 using HindIII digestion
pABB709	pABB708 with inserted ApaI cleavage site (PCR directed mutagenesis with primers #37 and #38)
pABB710	pABB709 with inserted MCS between ApaI-PvuII cleavage sites (annealed oligonucleotides #21 and #22)
pABB711	pABB709 with Km^R cassette replaced by modified version of Cm^R cassette originating from pABB705 using HindIII digestion
pABB712	pABB711 with inserted the first part of MCS between ApaI-PvuII cleavage sites (annealed oligonucleotides #17 and #18)
pABB713	pABB712 with inserted transcriptional terminator T_{tnp513} RA3 between KpnI-PagI restriction sites (annealed oligonucleotides #3 and #4)
pABB714	pABB713 with inactivated NcoI cleavage site in MCS by blunting
pABB715	pABB714 with inserted the second part of MCS between EcoRV-KpnI cleavage sites (annealed oligonucleotides #19 and #20)
pAMB2.4	Medium copy- number pMOB1.3.2 derivative (PCR-directed mutagenesis with primers #47 and #48)
pAMB2.5	High copy-number pMOB1.3.2 derivative (PCR directed mutagenesis with primers #49 and #50)
pAMB21.4	Medium copy- number **pABB21** derivative (PCR-directed mutagenesis with primers #47 and #48)
pAMB21.5	High copy-number **pABB21**derivative (PCR directed mutagenesis with primers #49 and #50)
pJSB1.24	pBGS18 with tra_{RA3} region cloned as EcoRI-SalI fragment, conjugative vector
pJSB13.9	pABB20 with $oriT_{RA3}$ inserted between SalI-NcoI sites, mobilizable vector
pJSB15.9	pABB22 with $oriT_{RA3}$ inserted between SalI-NcoI sites, mobilizable vector

Table(s)

Table 2. **The list of oligonucleotides used in this study** (restriction enzymes recognition sites are in bold, putative Rho-independent terminator sequences are underlined, modified nucleotides during site directed mutagenesis are shown in lowercase).

Oligo Abbrev	Oligonucleotides	Sequence
#1	*dKpnNco1*	CA**CTGCAGACATGT**A
#2	*dKpnNco2*	CATGT**ACATGTCTGCAG**TGGTAC
#3	*Ttnp513 RA3*	CATGT**GGTACC**TGAAGCCCCAACTGTTATCAGTTGGGGCTTTTTCTTGTCTGTTT**CCATGG**C
#4	*Ttnp513 RA3*	CATGG**CCATGG**AAACAGACAAGAAAAAGCCCCAACTGATAACAGTTGGGGCTTCA**GGTACC**A
#5	*Tnic RA3*	CATGT**GGTACC**GATAAAAAAACCGGCCTGTTGGCCGGTTTTTGTTTTCAG**CCATGGCTGCAG**
#6	*TtraC3 RA3*	CATGG**CTGCAGCCATGG**CTGAAAACAAAAAACCGGCCAACAGGCCGGTTTTTTTATC**GGTACC**A
#7	*TtraC3 RA3 IIo*	CATGT**GGTACC**CTGAAAACAAAAAACCGGCCAACAGGCCGGTTTTTTTATC
#8	*Tnic RA3 IIo*	CATGGATAAAAAAACCGGCCTGTTGGCCGGTTTTTGTTTTCAG**GGTACC**A
#9	*Tpap P1*	CATGT**GGTACC**GAAAGTGAAAATAAAAACATGCCGCAAGGCGCGGCATGTTTCCAATCAAT**CCATGG**C
#10	*TaspmgT P1*	CATGG**CCATGG**ATTGATTGGAAACATGCCGCGCCTTGCGGCATGTTTTTATTTTCACTTTC**GGTACC**A
#11	*TaspmgT P1 IIo*	CATGT**GGTACC**GATTGATTGGAAACATGCCGCGCCTTGCGGCATGTTTTTATTTTCACTTT**CCATGG**C
#12	*Tpap P1 IIo*	CATGG**CCATGG**AAAGTGAAAATAAAAACATGCCGCAAGGCGCGGCATGTTTCCAATCAATC**GGTACC**A
#13	*Tpro P1*	CATGT**GGTACC**AACCACCAAAAATAACCCCGGCAGCTGCCGGGGTTCTCGTTAACTATTAT**CCATGG**C
#14	*Tlyz P1*	CATGG**CCATGG**ATAATAGTTAACGAGAACCCCGGCAGCTGCCGGGGTTATTTTTGGTGGTTGGTACCA
#15	*Tlyz P1 IIo*	CATGT**GGTACC**ATAATAGTTAACGAGAACCCCGGCAGCTGCCGGGGTTATTTTTGGTGGTT**CCATGG**C
#16	*Tpro P1 IIo*	CATGG**CCATGG**AACCACCAAAAATAACCCCGGCAGCTGCCGGGGTTCTCGTTAACTATTAT**GGTACC**A
#17	*ApaPvuC1*	t**GATATCGGTACCTCATGAGCGGCCGC**CAG
#18	*ApaPvuC2*	CTG**GCGGCCGCTCATGAGGTACCGATATC**aGGCC
#19	*EcoKpnC1*	ATC**CTCGAGACATGTGTCGACTCATGAGGATCCCCATGG**aGTAC
#20	*EcoKpnC2*	t**CCATGGGGATCCTCATGAGTCGACACATGTCTCGAG**GAT
#21	*KApaPvu1*	a**TCGCGAGGATCCGTCGACGAATTCACATGTCTCGAGCCATGG**CAG
#22	*KApaPvu2*	CTG**CCATGGCTCGAGACATGTGAATTCGTCGACGGATCCTCGCGA**tGGCC
#23	*galK F*	ATGATCTTTCTTGCCGAGCG
#24	*galK R*	AGCAGCTTTATCATCTGCCGC
#25	*repB2F*	CATCGAGAAGCAAAAGGCG
#26	*repB2R*	CCAACTTGCGTAGGTCTTCCAG
	Primers used in site directed mutagenesis	
#27	*KmXhom1*	CGGGAAACGTCTTGtTCGAGGCCGCG
#28	*KmXhom2*	ATCGCGGCCTCGAaCAAGACGTTTC
#29	*KmNrum1*	GGGTATAAATGGGCaCGCGATAATGTCG
#30	*KmNrum2*	GCCCGACATTATCGCGtGCCCATTTATAC
#31	*CATNcom1*	CGCCCCCGTTTTCACgATGGGCAAATATTATAC
#32	*CATNcom2*	GCGTATAATATTTGCCCATcGTGAAAACGGGGG
#33	*CATPvum1*	CAGACCGTTCAGCTcGATATTACGGCC
#34	*CATPvum2*	AGGCCGTAATATCgAGCTGAACGGTC
#35	*mRA3Ecoa*	GATACTTGAAAGGGAgTTCTTGGCCCCG
#36	*mRA3Ecob*	GTACGGGGCCAAGAAcTCCCTTTCAAG
#37	*RA3ApaIa*	CGATACTCAAGACCgGGCCCATCAGC
#38	*RA3ApaIb*	CAGCTGATGGGCCcGGTCTTGAGTATC
#39	*19BclNca*	GGAAAGAACATGTGAtCAAAAGGCCAtggAAAGGCCAGGAACCG
#40	*19BclNcb*	GTTCCTGGCCTTTccaTGGCCTTTTGaTCACATGTTCTTTCCTG
#41	*RA3NotIa*	GCAGCTCGACCAGGcGGCCgcTTTCGTAC
#42	*RA3NotIb*	GTACGAAAgcGGCCgCCTGGTCGAGCTG
#43	*TcEcoRV1*	GGGCCTCTTGCGGGAcATCGTCCATTCCGAC
#44	*TcEcoRV2*	CTGTCGGAATGGACGATgTCCCGCAAGAGGC
#45	*PagTcmF*	GCCGAAACAAGCGCTCATaAGCCCGAAGTGGCGAG
#46	*PagTcmR*	GGCTCGCCACTTCGGGCTtATGAGCGCTTGTTTCGG
#47	*RepXm1*	GTTAGCGGCgTAGAAAGGGAgctCCCCGGAAACC
#48	*RepXm2*	TTCCGGGGagcTCCCTTTCTAcGCCGCTAACGG
#49	*opA1*	CTAGGTTACACTctAgAACACATCATTCTG
#50	*opA2*	GAATGATGTGTTcTagAGTGTAACCTAGTTG

Figure 1. Analysis of transcriptional terminators from P1 prophage and RA3 plasmid. (**A**) Schematic map of test vectors used in the read-through transcription assay *in vivo*. (**B**) Summary of the analysis of read-through transcription assay *in vivo*. Activity of the promoters tested in the presence of transcriptional terminator signals was monitored by catechol 2,3 oxygenase levels in the extracts from cells of the logarithmically growing cultures of appropriate transformants of DH5α strain. The results are shown as % of read-through transcription compared to the control - strain carrying plasmid without inserted terminator between the promoter and *xylE* gene (pABB70.1 or pABB58.1, respectively). The average value from three independent experiments is presented.

Figure 2. Vectors for cloning and shuffling of different gene cassettes in bacteria. Map of (**A**) pABB19, (**B**) pABB20, (**C**) pABB21, (**D**) pABB22 showing relevant properties of the vector. DR1, *repA*, *repB* and DR2 represent minireplicon of RA3 plasmid. The unique or double restriction sites important for cloning and cassette exchange are shown, the relevant sites described in the text are in bold. T1, T2 and T3 correspond to the transcription terminator sequences T_{pro}/T_{lyz} P1, T_{nic}/T_{traC3} RA3 and T_{tnp513} RA3, respectively. (**E**) Exemplary scheme of sequential cloning of independent gene cassettes using constructed vectors pABB21 and pABB22 (for details see the text).

Figure 3. Copy number and stability of RA3 minireplicon derivatives. (**A**) Plasmid DNA isolated from *E. coli* DH5α transformants with pABB21 and its derivatives pAMB21.4 and pAMB21.5 showing varied copy number. (**B** and **C**) Plasmid stability assays. RA3 minireplicon derivatives were propagated in *E. coli* DH5α (**B**) and *P. putida* KT2442 (**C**) for 40 generations without selective pressure. Plasmid stability was determined at the starting point (0) and after (20) and (40) generations of growth by replica plating onto selective and non-selective media and presented as a percentage of cells that retain antibiotic resistance. Each bar represents the average of three independent experiments.

Figure(s)

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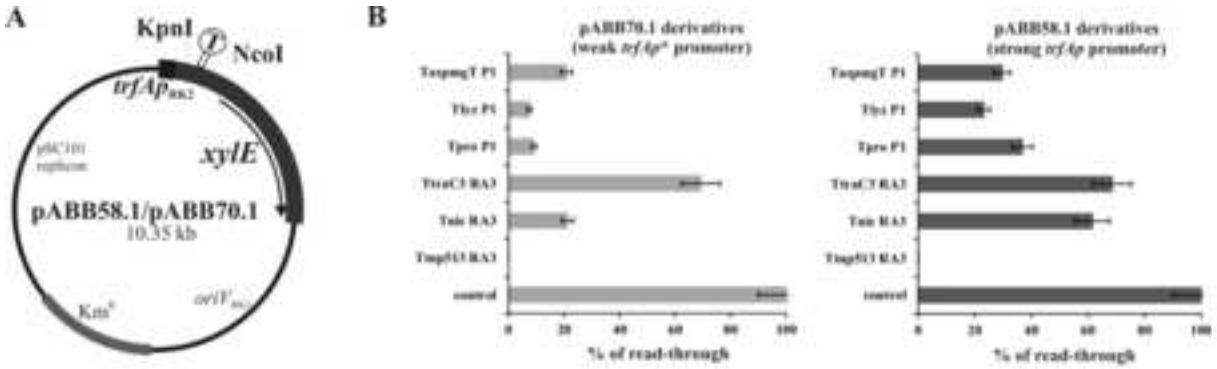

Figure 1

Figure(s)

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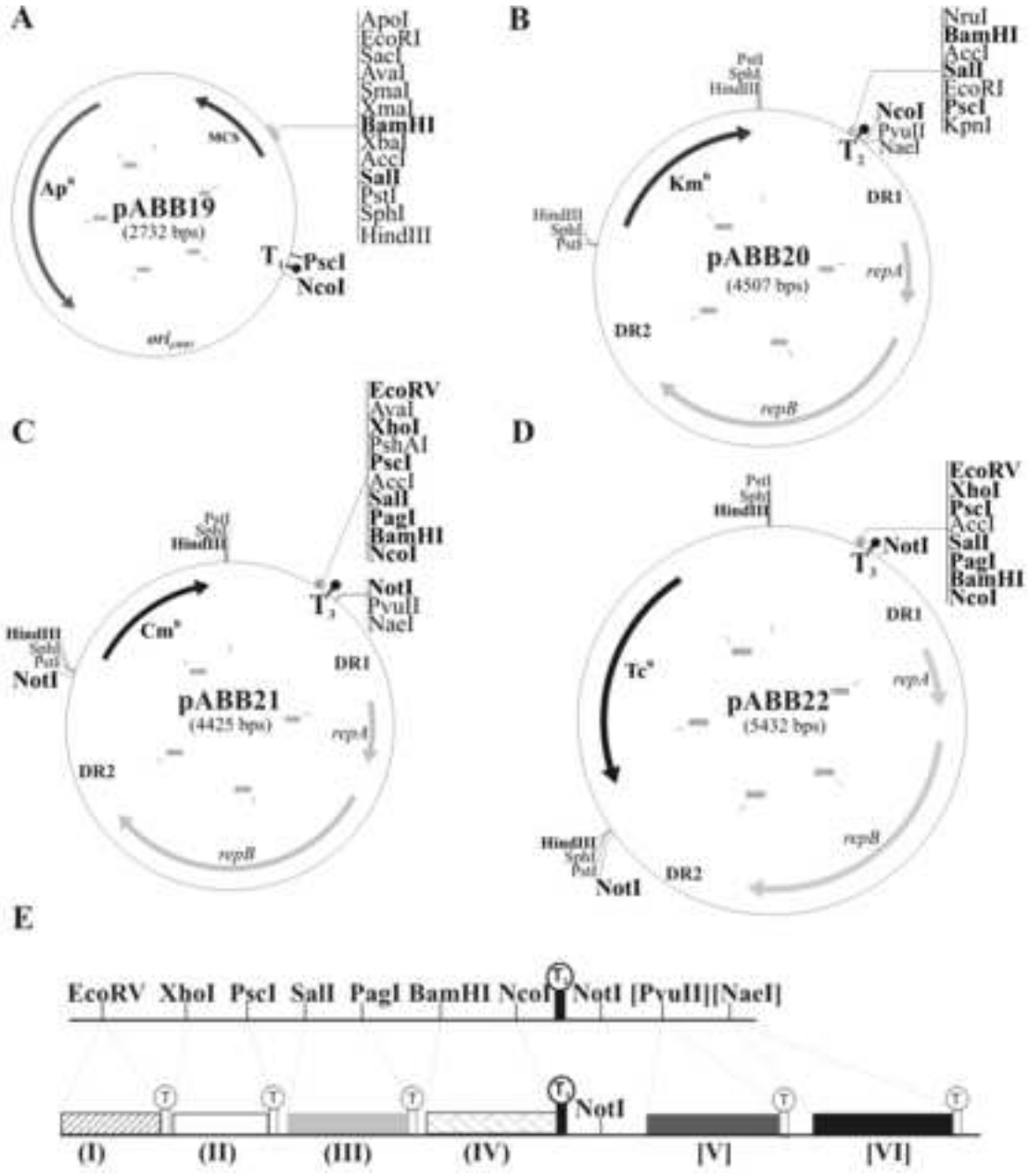

Figure 2

Figure(s)

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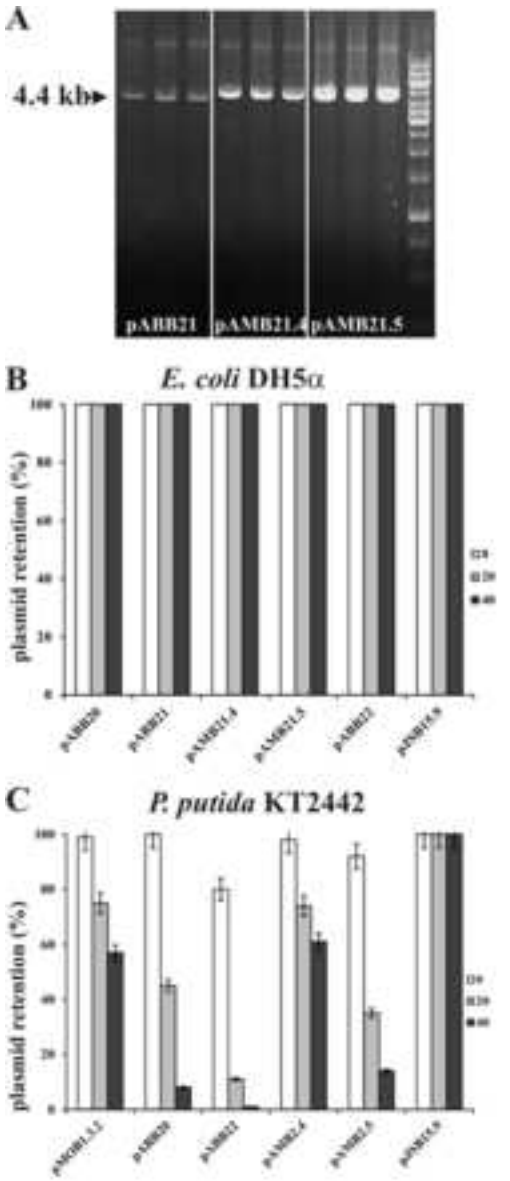

Figure 3