Convenient broad-host-range unstable vectors for studying stabilization cassettes in diverse bacteria

Aneta A. Bartosik, Krzysztof Glabski, Anna Kulinska, Ewa Lewicka, Jolanta Godziszewska, Aleksandra Markowska and Grazyna Jagura-Burdzy

Abstract

Background: Low-copy-number vectors of potential wide application in biotechnology need to encode stabilization modules ensuring their stable inheritance. The efficiency of stabilization may vary depending on the plasmid host so a thorough analysis of stabilization functions is required before use.

Results: To facilitate such analysis highly unstable, mobilizable, broad-host-range (BHR) vectors based on RK2 replicon were constructed. The vectors are suitable for testing of various stabilization functions, including plasmid and chromosomal partitioning cassettes encoding ParB homologues capable of spreading on DNA. The xylE or lacZ reporter systems facilitate easy monitoring of plasmid segregation.

Conclusion: The range of BHR vectors with different reporter cassettes and alternative mobilization systems expands their application in diverse bacterial species.

Keywords: Broad-host-range, Cloning vector, RK2, lacZ, xylE reporter, Stability functions

Background

The stabilization functions carried by low-copy-number plasmids from a wide range of bacteria ensure their stable inheritance during cell division [1]. Putative stabilization modules (e.g., partitioning operons, toxin-antitoxin systems, restriction-modification mechanisms) are also encoded on bacterial chromosomes [2–6]. Such modules could be used to construct vectors for biotechnological applications. The properties of the stabilization modules may vary depending on the host they are expressed in, so a thorough analysis is required before use.

Several test vectors are available for studying stabilization functions in bacteria. Most of them rely on narrow-host-range replicons and can be used only in certain E. coli strains or other narrowly defined hosts [7, 8]. pALA136 [9] and pOG04 [10] are based on dual pMB1 and P1 or P7 replicons, respectively. The high-copy-number pMB1 replicon requires PolI for replication, so the plasmid is stable in polA+ strains but when transformed into a polA mutant, it depends on the phage vegetative replication system and consequently becomes highly unstable as a single-copy molecule unless a stabilization cassette is included.

The standard method for testing putative stabilization functions relies on a classical segregation test, in which a strain with the plasmid is cultured for a certain number of generations without selection and then the number of cells still carrying the plasmid is estimated by the time-consuming replica plating of colonies or serial dilutions (drop test). Introduction of the reporter gene lacZ in pOU82 [11] simplifies the screening for plasmid loss, but this very useful test vector can only be applied for E. coli and its closely related species since it relies on the narrow-host-range R1 replicon of the IncFII incompatibility group [12].

This paper presents a set of highly unstable broad-host-range plasmids based on the RK2 replicon of IncP-1 group [13] designed to test stabilization functions in diverse bacterial species. pABB35 and its derivatives are single-copy vectors specifying chloramphenicol resistance (CmR). The multiple cloning site (MCS) is flanked by lacO operators...
serving as binding sites for LacI repressor to build a roadblock for polymerizing ParB-type proteins encoded by the tested partitioning cassettes of type IA [14, 15]. The unstable vectors contain the xylE (klcAPRA3·xylE-Tpro/lacP1) or lacZ (klcAPRA3·lacZ-Tpro/lacP1) reporter gene enabling easy and quick detection of bacterial colonies retaining the plasmid with the potential stabilization cassette. The plasmid segregation process can also be monitored in liquid cultures by a quantitative XyIE activity assay. Variants of the unstable vector mobilizable by the RK2 (IncP-1) conjugative system integrated into the E. coli chromosome or by the RA3 (IncU) one integrated into the Pseudomonas putida chromosome are also available.

Results and discussion
Construction of a highly unstable broad-host-range plasmid
The main aim of this project was to engineer an unstable cloning vector suitable for easy monitoring of segregation functions in a wide range of bacteria.

We chose pRK415 [16], a derivative of the RK2 replicon from the IncP-1a incompatibility group, to construct a highly unstable broad-host-range (BHR) test vector.

The pRK415 cloning vector contains four following RK2 fragments: i/ a region encoding Ssb (single-stranded DNA-binding protein), the replication initiator protein TrfA, Upf16.5 of unknown function [13], and TrbA, a regulatory protein of RK2 conjugative transfer operons [17]; ii/ oriV_RK2 with eight iterons constituting TrfA binding sites [18, 19]; iii/ part of the central control operon korA-incC encoding KorA, the primary repressor of trfAp [20], since strong trfAp is unclonable when unregulated [21], and iv/ the traJ traK intergenic region with oriT_RK2 to facilitate mobilization by the RK2 conjugation system [22]. Additionally, the vector carries a tetracycline resistance cassette (TcR) and a lacp-lacZ fragment with MCS for α-complementation and easy identification of cloned inserts. pRK415 had previously been reported as slightly unstable [16, 23], but our plasmid retention tests showed its almost 100 % stability, since after 40 generations of growth of E. coli DH5α (pRK415) under non-selective conditions in L broth without antibiotics almost 100 % of cells retained the plasmid (Fig. 2a).

The strategy to obtain a truly unstable derivative of the RK2 minireplicon was to limit the expression of trfA, first by introducing a promoter-down mutation in trfAp and, if required, by adding KorB, a second repressor acting cooperatively with KorA on trfAp [24], to the system. It has previously been shown that the T → C mutation in the -10 sequence of trfAp (trfAp-1) decreases the promoter activity at least 10-fold [17]. Site-directed PCR mutagenesis was used to introduce trfAp-1 mutation together with an AatII restriction site into pRK415. Plasmid DNA sequencing confirmed the introduction of the desired mutation into the -10 sequence of trfAp, but also an unexpected deletion of 1974 bp encompassing lacZα with MCS and the tral-traK region with oriT_RK2. The obtained 8716-bp derivative pAKB20.1 (Fig. 1a) was still very stably maintained in E. coli DH5α cells demonstrating 100 % retention after 40 generations of growth under non-selective conditions (data not shown).

To facilitate insertion of potentially large DNA fragments bearing stability cassettes it was required to downsize the cloning vector. Hence, pAKB20.1 was modified by NcoI digestion and self-ligation to delete a 527-bp fragment of the upf16.5 gene of unknown function [13], the last orf in the ssb-trfA-upf16.5 operon present only in one subgroup of IncP-1 plasmids (IncP-1a). Although an Upf16.5 function in copy number control (e.g., efficient replication initiation) has not been reported yet, the new derivative, pABB25, was slightly less stable in E. coli DH5α cells in comparison to pAKB20.1 and after 20 generations of growth without selection 20 % of cells lost the pABB25 plasmid (Fig. 2a). In the next step the tetracycline resistance operon (TcR) of pABB25 was replaced with a shorter cat gene (encoding chloramphenicol acetyltransferase) conferring the CmR phenotype. The resulting plasmid pABB25.1 (CmR) demonstrated stability in E. coli DH5α comparable to that of pABB25 (data not shown).

The initial manipulations did not sufficiently decrease the stability of the RK2 minireplicon, so it was decided to proceed with the addition of korB encoding a co-repressor of trfAp, to the system. It was also important to inactivate incC since IncC and KorB constitute the active partitioning system of RK2 [25, 26]. Two restriction sites, Apal and Xhol were introduced into pABB25 to facilitate substitution of incC2 orf with korB_RK2 and to give pABB27. Before korB cloning, a Smal-NcoI fragment encoding klcAPRA3·xylE-Tpro/lacP1 was inserted into pABB27 between the trfA and cat genes to give pABB28 (Fig. 1b). klcAPRA3 is a strong promoter and xylE encodes catechol 2,3- dioxygenase, whose activity is easy to be monitored following bacteria growth on plates [27] or in liquid cultures (this work). Subsequently, the korB_RK2 was inserted into pABB28 between the Xhol and Apal sites. The obtained pABB29 plasmid (Fig. 1c) demonstrated a high loss rate in E. coli DH5α strain (Fig. 2a), pABB29, comprising a modified RK2 replication system, the korA-korB operon, the klcAp-xylE-Tpro/lacP1 reporter cassette and the Cm resistance marker, was used in the next step to prepare the final version of the unstable BHR vector for cloning and testing stabilization cassettes in a wide range of bacterial species.

The MCS introduced between the unique NcoI and BssHII (Paul) sites in pABB29 to give pABB32 (Fig. 1d) contained BglII, EcoRI, SnaBI, Xhol, Eco11B6 and SacI restriction sites and was surrounded by lacO operator sequences. The binding of LacI repressor to the lacO operators was expected to act as a roadblock [28] for potentially
Fig. 1 Milestones in construction of unstable BHR vector. Circular maps of intermediate (a, b and c) and final vectors (d, e and f) are drawn to scale. Only intact orfs are indicated. Unique or double restriction sites important for cloning are shown, those described in the text are in bold. T₁ marks the divergent transcription terminator sequence T₁pro/T₁lyz of P1 prophage [49].
polymerizing ParB partitioning proteins encoded by type IA partition cassettes [14, 15] that might be analyzed using this vector. ParB spreading that follows its binding to parS (centromere-like sequence) may lead to transcriptional silencing of nearby genes and therefore affect results of segregation studies [29–33].

Finally, the lacP allele [34, 35] was inserted into pABB32 to obtain the final construct, the pABB35 vector (Fig. 1e). The lacP mutation refers to a change in the −35 motif of lacP causing overexpression of lacI (superrepressor) [36] and is often used in recombinant strains or vectors to provide tighter control of lacZp (or hybrid tacp) expression in the absence of the IPTG inducer.

The high instability of pABB35 was confirmed by the standard stability test: only approximately 2% of cells retained the plasmid after 20 generations of growth without selection (Fig. 2a).

**Plasmid copy number**
The copy number of chosen plasmid constructs described above was determined in *E. coli* cells by qPCR [37]. The pRK415 derivatives pAKB20.1, pABB25 and pABB29 were present in 4 to 6.5 copies per chromosome, similarly to pRK415 itself (Fig. 2b). The number of pABB32 and pABB35 copies was 1.5 - 2.5 per *E. coli* chromosome. This lower copy number is due to the tight regulation of trfA-1 and underlies the instability of these test vectors. The copy number of pMPB9.90 araBADp-trfA base of pBAD24 [38], was established at 50 copies per chromosome in correlation with a published data [39]. For comparison also a single-copy-number plasmid RA3 of IncU group [40] was used and demonstrated 1–2 copies per chromosome (Fig. 2b).

![Fig. 2 Standard stability tests of constructed vectors. *E. coli* DH5α transformants were grown overnight with antibiotic (point 0) and for 40 generations without antibiotic. Every 20 generations appropriate dilution was plated on L agar to obtain approximately 100 colonies. The colonies were replica plated to test for chloramphenical resistance. Plasmid retention was expressed as percentage of CmR colonies. The results shown are average from three experiments with standard deviation. a Retention of constructed vectors. b Plasmid copy number estimated by RealTime qPCR. Plasmid copy relative to the chromosome was assayed in three independent biological samples with three technical replicates each. Average results for plasmids are presented with SD as follows: 0.06; 1.75; 0.21; 0.16; 0.95; 0.32; 0.17; 6.96, respectively. c Stabilizing properties of active partitioning operon from RA3 in pABB32 and pABB35 vectors. d Effect of IPTG-induced expression of *P. aeruginosa parA-parB* operon on pABB34 plasmid retention. DH5α(pABB34) cultures were grown in L broth with various concentrations of IPTG.

Plasmidic and chromosomal partitioning cassettes stabilize test plasmids in *E. coli*
The type IA active-partitioning cassette korA-incC-korB-orf1-parS from RA3 plasmid [40, 41] was chosen to check the applicability of the constructed vectors in a stabilization assay in bacteria. The cassette was cloned into pABB32 and pABB35 vectors to obtain pAKB17.9 and pAKB17.10, respectively, and both plasmids were tested in the standard stabilization assay in *E. coli* DH5α strain.
The RA3 partitioning cassette did not drastically improve the pABB32 plasmid segregation rate: approximately 10 % of cells retained the pAKB17.9 plasmid after 20 generations of growth without selection (Fig. 2c). Remarkably, the same RA3 fragment cloned into pABB35 exhibited the expected stabilization function and pAKB17.10 was retained in approximately 70 and 60 % of E. coli DH5α cells after 20 and 40 generations of growth without selection, respectively (Fig. 2c). The only difference between pABB32 and pABB35 is the presence of the lacP allele in the latter (Fig. 1d and e). Since it has been shown previously that KorB_R3 (a ParB homolog) spreads on DNA after binding to parS and silences nearby genes [41], the different stability of pABB17.9 and pAKB17.10 convincingly demonstrates that overproduction of LacI and its binding to lacO sequences flanking the cloned stabilization cassette blocks effectively the KorB spreading.

The usefulness of the constructed vectors was also checked with a synthetic chromosomal partition cassette lacP-tacp-parA-parB-parS from P. aeruginosa [29] cloned into pABB32 to give pABB34.

The pABB34 plasmid was present in more than 50 % of E. coli cells after 20 generations of growth without selection, in comparison to only 10 % of cells retaining empty pABB32 (Fig. 2d). After 40 generations pABB34 was still present in 14 % of cells, whereas the retention of pABB32 dropped below 1 %.

The stabilization effect of the RA3 partitioning cassette cloned in pAKB17.10 was stronger than that demonstrated by the parA-parB-parS_pa, cassette present in pABB34 (Fig. 2c and d). These differences in the stabilization potential could reflect the individual properties of each cassette, but the rather modest effect of the synthetic parA-parB-parS cassette of P. aeruginosa could also be due to the low amount of partitioning proteins produced since the parA-parB operon in pABB34 is expressed at a low basal level from the strongly repressed tacp. To check which explanation was correct, different concentrations of IPTG were used to boost the production of the partition proteins ParA and ParB. In support of the latter possibility IPTG at 0.02–0.2 mM improved the stability of pABB34 (Fig. 2d). A further increase in IPTG concentration (0.5 mM) did not improve the plasmid stability (data not shown) probably due to the antagonistic effect of IPTG on the action of LacI as the roadblock to ParB, known to spread on DNA starting from parS [29].

Catechol 2,3-dioxygenase activity assay as an estimate of plasmid stability

The results presented in Fig. 2 come from a standard stabilization assay with the use of replica plating to estimate the proportion of colonies retaining the plasmid tested (in this case, conferring resistance to chloramphenicol). The xylE reporter cassette present in pABB32 and pABB35 allows the plasmid segregation to be assayed using a simple plate test to visualize colonies that express the xylE reporter gene and hence must have retained the plasmid.

The cultures of transformants were grown without selection for a certain number of generations, diluted and plated onto L broth without antibiotics to get 100 to 200 colonies. The colonies were sprayed with 10 mM catechol and those derived from cells that had lost the test plasmid with xylE remained opalescent, those in which the test plasmid was stably maintained turned yellow quickly (Fig. 3a), whereas colonies of strains carrying an unstable plasmid with the xylE gene, were in various shades of yellow.

A quick semi-quantitative test for plasmid stability can also be performed for liquid cultures directly. Addition of catechol to overnight cultures (2x10⁶ cells ml⁻¹) to 1 mM final concentration clearly distinguishes those in which XylE is produced by the majority of cells (the test plasmid is stably maintained) from the ones where the plasmid is hardly retained (high plasmid loss rate) (Fig. 3b). Care must be taken to determine the initial rate of reaction, i.e., to measure OD₄₁₅ within a few minutes (2–5) after substrate addition [27, 34, 42].

Comparison of the standard plasmid stability assay with plate catechol 2,3-dioxygenase determination

E. coli DH5α transformants bearing appropriate test plasmids were cultivated in L broth without antibiotics as earlier and tested for plasmid retention after approximately 20, 40 and 60 generations using, in parallel, the standard stabilization assay and the xylE plate test described above. As shown in Fig. 3c the results of plasmid retention estimation are quite similar for the two assays, justifying the use of the quicker xylE test.

High-throughput analysis of plasmid stabilization functions

Plasmid retention was quantified in liquid cultures of DH5α(pAKB17.10) and DH5α(pABB34) using a high-throughput procedure. In this experiment the production of ParA and ParB in DH5α(pABB34) was not induced by IPTG to have two plasmids (pABB34 and pAKB17.10) stabilized to different extent by the various partition cassettes [compare Fig. 2c and graph marked pABB34 (no IPTG) in Fig. 2d]. DH5α(pABB35) and DH5α(pESB32) strains were used as controls. Cultures of the transformants were grown overnight under selection (10 μg ml⁻¹ chloramphenicol) and then diluted to 5 cells ml⁻¹ in L broth and aliquoted into 100-well plates (200 μl per well). The plates were incubated at 37 °C with shaking for ca. 20 generations. In parallel the overnight start cultures were diluted 10⁵-fold and grown in tubes for 20 generations without selection and then diluted, aliquoted and incubated as above (a total of 40 generations without selection). Overnight subcultures
derived from single cells and grown in the 100-well plates were diluted 100-fold, OD\textsubscript{600} values were measured and after addition of 10 mM catechol to each well (final concentration 1 mM) OD\textsubscript{405} was read after 10 min. The results obtained after 20 generations for DH5\textalpha\(\text{(pABB35)}\) showed an almost complete lack of the plasmid indicating...
its high instability (only ca. 1.5 % of wells showed OD405 above background values obtained for the strain bearing pESB32 without xylE, data not shown). In the case of DH5α(pABB34) and DH5α(pAKB17.10) XylE activity was clearly detectable in 96 and 90 % of wells, respectively, after 20 generations of growth without antibiotic (Fig. 3d). After 40 generations of growth without antibiotic the corresponding values were 65 and 82 % for DH5α(pABB34) and DH5α(pAKB17.10), respectively (Fig. 3f). These results reflected well the differences in stability of the two plasmids observed in the standard and colony visualization assays when DH5α(pABB34) was grown without IPTG (Fig. 2d).

To normalize obtained data the OD405/OD600 ratio was calculated. It was around 2–3 for the control DH5α(-pESB32) strain and varied between 2 and 50 for the DH5α(pABB34) and DH5α(pAKB17.10) strains (Fig. 3e and g). When the ratios were plotted and analyzed, the medians reflecting plasmid retention rates in the subcultures were similar for the two plasmids following growth without selection for 20 generations, and substantially higher for DH5α(pAKB17.10) compared with DH5α(pABB34) after 40 generations of growth without selection, as observed before.

The high-throughput approach is obviously more reliable than the standard and colony visualization method since human error is minimized and such a quantitative procedure may help to demonstrate even small, but statistically significant differences in stabilities between various plasmids in a given host, the same plasmid in various hosts, or different plasmids with cloned partitioning operons in three different hosts. The use of pESB34 with pESB32 or pESB34 (korA-incC-korB-orf11-parS) and transformants were used to estimate plasmid retention (Fig. 4a). pESB32 or pESB34 were also used to transform E. coli S17-1 and transformants were applied as donors in conjugation with a RifR derivative of A. tumefaciens LBA1010R. The transconjugants were grown under selective conditions, then diluted appropriately and plated on L agar with X-gal. The retention rates of both plasmids assessed by the number of blue colonies are shown in Fig. 4b. pESB32 was less unstable in both strains tested in comparison with the original pABB35 in E. coli DH5α (Fig. 2a) probably due to variations in the functioning of the copy-number control circuit of the RK2 minireplicon. The presence of the RA3 partition cassette still significantly increased the pESB34 retention in P. aeruginosa PAO1161 strain but had a much lower impact on plasmid stability in A. tumefaciens LBA1010R. The reasons for the observed differences in empty vector stability and the stabilization effects of a given cassette in various bacterial species await elucidation.

**Modifications of the test vectors to expand their applicability**

The test plasmid pABB35 based on the RK2 minireplicon can propagate in a variety of species. Since many bacterial species are not easily transformable, two different oriT regions amplified from BHR conjugative plasmids, RK2 and RA3, were inserted additionally to pABB35 to give pESB30 and pESB31, respectively. Such vectors are mobilizable during conjugation of the recipient with two different bacterial species, E. coli S17-1 with integrated RK2 plasmid [43] or P. putida KT2440 with integrated conjugation module of RA3 (KT2440 traRA3), which may significantly extend the range of recipient strains.

The suitability of pESB30 vector for investigating stability mechanisms other than active partition was in the meantime confirmed by D. Bartosik’s group studying plasmidic toxin-antidote (TA) systems. The use of pESB30 vector with cloned hipAB system (TA) of pKON1 from Paracoccus kordatitaeve [44] allowed analysis of its stabilization functions in various species of Alphaproteobacteria e.g., P. pantotrophus and Ochrobactrum sp. (Czarnecki and Bartosik, personal communication).

Quick identification of colonies carrying the constructed vectors with the use of the color reaction enabled by xylE cassette could not be applied to some analyzed species e.g., P. aeruginosa, since when PAO1161 was transformed with xylE plasmids it formed yellow colonies due to the intrinsic substrates for catechol 2,3-dioxygenase. An alternative test vector, pESB32 (Fig. 1f), was constructed with the klcAp-lacZ cassette enabling monitoring of plasmid presence by formation of blue colonies in the presence of X-gal.

The partition operon korA-incC-korB-orf11-parS of RA3 was inserted between the EcoRI and XhoI sites in pESB32 to give pESB34. P. aeruginosa PAO1161 was transformed with pESB32 or pESB34 (korA-incC-korB-orf11-parS) and transformants were used to estimate plasmid retention (Fig. 4a). pESB32 or pESB34 were also used to transform E. coli S17-1 and transformants were applied as donors in conjugation with a RifR derivative of A. tumefaciens LBA1010R. The transconjugants were grown under selective conditions, then diluted appropriately and plated on L agar with X-gal. The retention rates of both plasmids assessed by the number of blue colonies are shown in Fig. 4b. pESB32 was less unstable in both strains tested in comparison with the original pABB35 in E. coli DH5α (Fig. 2a) probably due to variations in the functioning of the copy-number control circuit of the RK2 minireplicon. The presence of the RA3 partition cassette still significantly increased the pESB34 retention in P. aeruginosa PAO1161 strain but had a much lower impact on plasmid stability in A. tumefaciens LBA1010R. The reasons for the observed differences in empty vector stability and the stabilization effects of a given cassette in various bacterial species await elucidation.

**Conclusions**

We have manipulated the broad-host-range RK2 minireplicon pRK415 to obtain a highly unstable pABB35 vector. To facilitate easy monitoring of plasmid retention, a xylE or lacZ reporter system was inserted. A multiple cloning site surrounded by roadblocks for protein spreading enables analysis of various partition cassettes. To broaden applications of the vector to a variety of hosts we added oriT regions from RK2 or RA3 BHR plasmids so they could be mobilized by the widely used S17-1 (with RK2 integrated) and P. putida KT2440 with the tra module of RA3 constructed in this work, respectively.

The constructed vectors were demonstrated to be useful for cloning chromosomal and plasmid partition operons that produce type IA ParB-like proteins able to spread on DNA. Retention of the vectors varied depending on the host so they need to be tested in a given strain prior to application. Analysis of the stabilization properties of the cloned partitioning operons in three different hosts (E. coli, P. aeruginosa, A. tumefaciens) confirmed their variability and at the same time necessity to conduct such experiments.
Estimation of plasmid stability using catechol 2,3-dioxygenase assay in liquid cultures facilitates large scale or high-throughput experiments in bacteria, since hundreds or even thousands of variants can be monitored easily. It can be used to screen for new stabilization cassettes in meta-genomic approaches, to study stability functions in diverse bacteria and to screen mutant proteins affecting plasmid stability as well as inhibitors of the systems.

The test plasmids described here with an easy detection/monitoring system should be useful for studies of various plasmid stability as well as inhibitors of the systems.

Methods
Bacterial strains and growth conditions
Diplococcus pneumoniae strains used were DH5α [F’(Φ80lacZ ᵃ ᵃ recA1 endA1 gyrA96 thi-1 hsdR17(rKm²) supE44 relA1 deoR Δ(lacZYA-argF)U169]) and S17-1 [recA pro hsdR RP4-2-Tc::Mu-Km::Tn7] [43]. DH5α RifR mutant was selected during growth on L agar with 150 μg ml⁻¹ rifampicin. P. putida KT2440 was kindly provided by C.M. Thomas (Birmingham University, Birmingham, United Kingdom). P. putida KT2440 harbors the trnAα parS operon to downsize the vector and to stabilize functions in a wide range of strains in which the RK2 replicon can propagate.

P. aeruginosa PAO1161 (leu, r+, m+) was kindly provided by B.M. Holloway (Monash University, Clayton, Victoria, Australia). Agrobacterium tumefaciens LBA1010R RifR was kindly provided by D. Bartosik (University of Warsaw, Warsaw, Poland). Bacteria were generally grown in L broth [46] at 37 °C or 28 °C (A. tumefaciens). L broth and L agar (L broth with 1.5 % w/v agar) were supplemented with appropriate antibiotics: chloramphenicol (10 μg ml⁻¹ for E. coli, 50 μg ml⁻¹ for A. tumefaciens and 150 μg ml⁻¹ for P. aeruginosa), kanamycin (50 μg ml⁻¹ for E. coli and P. putida) or tetracycline (10 μg ml⁻¹ for E. coli).

Strains with plasmids carrying the klcA-lacZ fusion were tested on L agar with 40 μg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Plasmid analysis and PCR amplification
Plasmid manipulations were carried out by standard procedures [47]. All plasmids constructed in this work are listed in Table 1.

Standard PCR [48] was performed with pairs of primers listed in Table 2.

PCR-based site-directed in vitro mutagenesis was performed with mutagenic primers (Table 2) as described previously [49]. The PCR mixture after mutagenesis was treated for 1-2 h with 10 U of DpnI restriction enzyme in order to eliminate template DNA and was used for transformation of E. coli DH5α.

All new plasmid constructs were verified by sequencing at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, using dye terminator sequencing and an ABI 377 Perkin Elmer automated sequencer. Sequences were analyzed using Clone Manager 9.

Plasmid construction
Construction of unstable BHR vector
Site-directed PCR mutagenesis with primers #18 and #19 introducing the T → C mutation in the -10 motif of trfA together with an AatII restriction site into prK415 was carried out to give pAKB20.1 (Fig. 1a).

pAKB20.1 was modified further by NcoI digestion and self-ligation to delete a 527-bp fragment of the upf16.5 gene from trfA operon to downsize the vector and to give pABB25.
| Name      | Relevant features                                                                 | References |
|-----------|-----------------------------------------------------------------------------------|------------|
| pABB19    | oriV<sub>MB1</sub>, Ap<sup>R</sup>, derivative of cloning vector pUC19 with added transcription termination sequence T<sub>pro</sub>/T<sub>lyz</sub> from P1 | [49]       |
| pABB705   | pKRP10 derivative with inactivated Ncol and PvuII sites in Cm<sup>R</sup> cassette | [49]       |
| pALA136   | oriV<sub>MB1</sub>, oriV<sub>P1</sub>, Cm<sup>R</sup>, dual replicon               | [9]        |
| pBG518    | oriV<sub>MB1</sub>, Km<sup>R</sup>, cloning vector                               | [56]       |
| pBAD24    | oriV<sub>MB1</sub>, Ap<sup>R</sup>, araC, araBAD<sup>p</sup>, expression vector   | [38]       |
| pCM132    | oriV<sub>ColE1</sub>, oriV<sub>Reo</sub>, oriR<sub>Q</sub>, tra<sup>T</sup>, tra<sup>A</sup>, Km<sup>R</sup>, promoter-less lacZ, dual replicon | [52]       |
| pGBT30    | oriV<sub>MB1</sub>, Ap<sup>R</sup>, lac<sup>F</sup>, tac<sup>p</sup> expression vector | [34]       |
| pGEM-T-Easy | oriV<sub>MB1</sub>, Ap<sup>R</sup>, cloning vector                               | Promega    |
| pJSB1.24  | pBG518 tra<sub>RA3</sub> korC<sub>RA3</sub> (RA3 coordinates 9437-33857; 3093-3705) | [49]       |
| pKLB3     | tacp-parA parB<sub>P.a</sub>                                                    | [29]       |
| pKRP10    | oriV<sub>MB1</sub>, Ap<sup>R</sup>, Cm<sup>R</sup>                                | [50]       |
| pMP89.90  | pBAD42 araBAD<sup>p</sup>-trf<sub>RA3</sub>                                        | Przyluski M. |
| pPT01     | oriV<sub>PacI</sub>, Km<sup>R</sup>, promoter-less xylE                          | [10]       |
| pRK145    | oriV<sub>Reo</sub>, Tc<sup>R</sup>, oriR<sub>Q</sub>, stable vector             | [16]       |
| pYC16A    | pAL136 with RA3 stabilization region                                             | [41]       |
| RA3       | IncU, Km<sup>R</sup>, Sm<sup>R</sup>, Su<sup>R</sup>                             | [40]       |
| RK2       | IncP-1<sup>q</sup>, Km<sup>R</sup>, Ap<sup>R</sup>, Tc<sup>R</sup>             | Thomas C.M. |

Plasmids constructed during this work:

| Name      | Description, relevant features                                                                 |
|-----------|---------------------------------------------------------------------------------------------------|
| pABB18.1  | pPT01 klcAp -xylE, PCR fragment amplified with primers #1 and #2 on RA3 template inserted as Sphl-BamHI fragment |
| pABB18.2  | pABB18.1 cleaved with Hpal and Ncol, filled in and self-ligated to remove 561 bp upstream of xylE |
| pABB18.3  | pABB19 with klcAp-xylE inserted as SmaI-Pscl PCR fragment amplified with primers #3 and #4 on pABB18.2 template |
| pABB18.4  | pBG518 with kor<sub>BA2</sub> inserted as EcoRI-Sall PCR fragment amplified with primers #5 and #6 on RK2 template |
| pABB18.5  | pGEM-T-Easy with lac<sup>R</sup> gene PCR-amplified primers #11 and #12 on GBT30 template |
| pABB25    | pAKB20.1 with 527-bp Ncol fragment removed                                                      |
| pABB25.1  | pABB25 with cat gene (Cm<sup>R</sup>) on BamHI fragment from pABB705 replacing Bcl fragment from Tc<sup>R</sup> cassette |
| pABB26    | pABB25.1 with Xhol restriction site introduced downstream of trbA (PCR directed mutagenesis with primers #20 and #21) |
| pABB27    | pABB26 with Apal restriction site downstream of kor<sub>A</sub> gene (PCR directed mutagenesis with primers #22 and #23) |
| pABB28    | pABB27 with klc-p-xylE cassette, Smal-Ncol fragment from pABB18.3                            |
| pABB29    | pABB28 with kor<sub>BA2</sub> gene, Apal-Sall fragment from pABB18.4 inserted between Apal and Xhol sites |
| pABB32    | pABB29 with MCS flanked with lacO operators inserted between BssHIII and Ncol sites, unstable vector |
| pABB33    | pABB32 with lac<sup>R</sup>-tacp-parA-parB<sub>P.a</sub>, Dral-Sall fragment of pKLB3 inserted between SnaBI and Xhol sites |
| Plasmid   | Description                                                                 |
|-----------|-----------------------------------------------------------------------------|
| pABB34    | pABB33 with parS<sub>par</sub>, annealed oligonucleotides #9 and #10 inserted into BglII restriction site |
| pABB35    | pABB32 with lacI<sup>q</sup>, NruI-PvuI fragment of pABB18.5 cloned between Smal and PvuI sites, unstable vector |
| pAKB17.9  | pABB32 with RA3 active partition cassette (korA-incC-korB-orf11-parS), EcoRV-BamHI fragment from pYC16A inserted between Ecl136II and BglII sites |
| pAKB17.10 | pABB35 with RA3 active partition cassette (korA-incC-korB-orf11-parS), EcoRV-BamHI fragment from pYC16A inserted between Ecl136II and BglII sites |
| pAKB20.1  | pRK415 T<sup>c</sup> with trfAp-1 introduced by PCR mutagenesis with primers #18 and #19 and spontaneous deletion of 1974-bp fragment encompassing MCS, traJ and oriT |
| pESB3.6   | pUC18 with synthetic RA3 partition cassette (korA-incC-korB-orf11-parS), cloned between EcoRI and SalI sites (RA3 coordinates 5940-9800) |
| pESB30    | pABB35 with oriT<sub>Hrc</sub>, 218-bp fragment PCR-amplified on RK2 template using primers #13 and #14, cleaved with PscI and cloned into Ncol site, unstable, RK2 mobilizable vector |
| pESB31    | pABB35 with oriT<sub>RAL</sub>, 166-bp fragment PCR-amplified on RA3 template using primers #15 and #16, cleaved with PscI and cloned into Ncol site, unstable, RA3 mobilizable vector |
| pESB32    | pESB30 with klcAp-lacZ, BglII-Ncol fragment from derivative of pCM132 inserted between BamHI and PscI sites, unstable, RK2 mobilizable vector |
| pESB34    | pESB32 with synthetic RA3 partition cassette (korA-incC-korB-orf11-parS), EcoRI-Sall fragment from pESB3.6 cloned between EcoRI and Xhol sites |
| pJSB1.28  | pJSB1.24 Ppu618, 618-nt PCR-amplified fragment of P. putida chromosome, coordinates S8074-S8691 |

Vectors for cloning and analysis of stabilization cassettes are in bold.
In the next step the tetracycline resistance operon (Tc\textsuperscript{R}) of pABB25 was replaced with a shorter cat gene (encoding chloramphenicol acetyltransferase) conferring the Cm\textsuperscript{R} phenotype. The BamHI fragment with the Cm\textsuperscript{R} cassette from pABB705 [49], a derivative of pKRP10 [50], with Ncol and PvuII restriction sites eliminated was inserted between the BclII sites to give pABB25.1.

Site-directed mutagenesis was used to introduce a unique Xhol site downstream of trb\textalpha (primers #20 and #21) and an Apal site downstream of kor\textalpha in the kor\textalpha-incC operon (primers #22 and #23) to give pABB27. kor\textalpha overlaps the 5' end of incC1 (incC encodes two forms of partition protein IncC1/IncC2 with two translational starts [51]) but is translated in a different reading frame, hence the mutagenic primers #22 and #23 introduced a stop codon precluding IncC1 translation.

The new vector pABB27 was further modified by insertion of the reporter cassette klcAp-xylE between the trf\textalpha and cat genes to give pABB28 (Fig. 1b).

The kor\textbeta\textbeta\textgamma\textalpha\textbeta\textdelta\textkappa gene was PCR-amplified with primers #5 and #6 on total DNA from E. coli DH5\textalpha(RK2) and inserted as a Sall-Apal fragment into pABB28 digested with Xhol and Apal to give pABB29 (Fig. 1c).

The unique Ncol and BssHII (PauI) restriction sites in pABB29 were used to introduce a new MCS sequence made from annealed oligonucleotides #7 and #8, yielding pABB32 (Fig. 1d).

lac\textbeta was amplified on pGBT30 [34] using primers #11 and #12 and inserted as NruI-PvuI fragment into pABB32 between Nal and PvuI sites to obtain the final pABB35 vector (Fig. 1e).

PCR-amplified 218-bp ori\textdelta\textkappa\textgamma\textkappa on RK2 template (primers #13 and #14) and 166-bp ori\textdelta\textkappa\textgamma\textkappa PCR-amplified on RA3 template (primers #15 and #16), were inserted as PscI fragments into the unique Ncol site of pABB35 to give mobilizable vectors pESB30 and pESB31, respectively.

II/Construction of klcAp\textbeta\textkappa-xylE and klcAp\textbeta\textkappa-lac\textbeta reporter cassettes

Two reporter genes, xylE of pWWO from P. putida encoding catechol 2,3-dioxygenase [10], and promoter-less lac\textbeta from pCM132 coding for β-galactosidase [52], were cloned into appropriate vectors.

The strong promoter klcAp [40] was PCR-amplified on RA3 template using primers #1 and #2 and cloned as an SphI-BamHI fragment into pPT0I vector [10] upstream of the xyl operon to yield pABB18.1. Subsequently, pABB18.1 was cut with Hpal and Ncol, filled in with PfuI Klenow fragment and self-ligated to remove a 561-bp fragment upstream of the xylE gene (pABB18.2). klcAp-xylE was PCR-amplified from pABB18.2 (primers #3 and #4) and cloned as a SmaI-PvuI fragment into pABB19 [49] to provide a cassette with a bi-directional Rho-independent transcription terminator, giving pABB18.3. The role of the transcription terminator inserted at the end of the reporter gene cassette was to prevent transcriptional spillover from the strong klc\textgamma promoter and to protect the klcAp-xylE cassette against transcription coming from inserts in the constructed vectors, as a new MCS was planned to be cloned next to the Ncol site. The Smal-Ncol fragment encoding klcAp\textbeta\textkappa-xylE-T\textsubscript{pro/OxyD1} was inserted into pABB27 to give pABB28.

pCM132 [52] carrying promoter-less lac\textbeta Z orf was cut with SphI and ligated with self-annealed oligonucleotide #17 to remove the SphI and insert an Ncol restriction site downstream of lac\textbeta (pCM132Nco). The BglIII-Ncol fragment from pCM132Nco carrying a promoter-less lac\textbeta cassette was inserted into pESB30 between the BamHI and Pscl sites to replace xylE and transcriptionally fuse lac\textbeta to the strong klc\textgamma promoter and to construct pESB32 (Fig. 1f).

III/Cloning the partitioning cassettes into the test vectors

Chromosomal parA-parB operon of P. aeruginosa had been cloned earlier under the control of tacp and lac\textbeta\textgamma in pKLB3 [29]. The DraI-Sall fragment of pKLB3 bearing lac\textbeta\textgamma-tacp-parA-parB was re-cloned between the SmalBI and Xhol sites of pABB32 (Fig. 1d), yielding pABB33. A centromere-like sequence parS\textgamma [29] made from annealed oligonucleotides #9 and #10 (Table 2) was cloned into BglIII-cut pABB33 to give pABB34 with the complete stabilization cassette from P. aeruginosa.

The EcoRV-BamHI fragment of pYC16A carrying the active partition cassette kor\textalpha-incC-kor\textbeta-orf11-par\textgamma from RA3 plasmid [40, 41] was cloned into pABB32 and pABB35 vectors digested with Ecl136II and BglII to obtain pAKB17.9 and pAKB17.10, respectively. In the case of pESB34, the EcoRI-Sall fragment from pESB3.6 carrying kor\textalpha-incC-kor\textbeta-orf11-par\textgamma of RA3 was cloned between the EcoRI and Xhol sites of pESB32.

IV/Construction of P. putida KT2440 traRA3 korRA3 Km\textsuperscript{R} helper strain

pJSB1.28 is a high copy number plasmid based on pMB1 replicon unable to replicate in P. putida. It is derivative of pJSB1.24 that carries the conjugative transfer module of plasmid RA3 (RA3 coordinates 9437-33857) together with the kor\textgamma gene (RA3 coordinates 3093-3705) encoding an indispensable transcriptional repressor [49]. A short region of P. putida KT2440 chromosome (coordinates 58074-58691) was PCR-amplified with the use of primers #24 and #25 (Table 2) and cloned between PstI and HindIII sites to facilitate integration of pJSB1.28 into the chromosome by homologous recombination. DH5\alpha (pJSB28) donor strain was conjugated with P. putida KT2440 and integrants were selected on M9 plates [47] with 0.1 % glucose and kanamycin (50 μg ml\textsuperscript{-1}) without thiamine to eliminate DH5\alpha that requires thiamine to
grow on minimal medium. The integration of pJSB1.28 into the chromosome of KT2440 was verified by PCR.

Transformation and conjugation procedures
Competent E. coli and P. putida cells were prepared by the standard CaCl$_2$ method [47]. Competent cells of P. aeruginosa were prepared according to the method of Irani and Rowe [53]. E. coli S17-1 strain was transformed with test plasmids carrying $oriT$$_{RK2}$ and such transformants were used as donors in conjugation with recipient A. tumefaciens LBA1010R Rif$^R$ strain. Overnight cultures of the donor and recipient strains (100 μl each) were mixed on L agar plate and incubated overnight at 28 °C. Bacteria from the plate were suspended in L broth and serial dilutions of the suspension were plated on L agar with rifampicin (150 μg ml$^{-1}$) and chloramphenicol (50 μg ml$^{-1}$) and incubated at 28 °C. Alternatively, P. putida KT2440 $tra_{RA3}$-$korC$ Km$^R$ strain was transformed with pABB35 derivatives carrying $oriT$$_{RA3}$ (e.g., pESB31) and such transformants were used as donor strains.

Table 2 Oligonucleotides used in this study

| No | Name | Sequence |
|----|------|----------|
| 1  | klcA$p_{RA3L}$ | GGCAGATGCGGGAGCGTGATCGTTACGGT |
| 2  | klcA$p_{RA3R}$ | GGCAGATCCATTGACGACATAGGGCGAGG |
| 3  | klcAsmA | GGCAGATGCGTGATCTGTCGATGTCGTC |
| 4  | xylEPscR | GGCACATGTGACATGCAATAATCTCAG |
| 5  | KBRK2ApA | GGCAGATGCGTGATCTGTCGATGTCGTC |
| 6  | KBRK2sal | GGCAGATGCGTGATCTGTCGATGTCGTC |
| 7  | lacOMCS1 | CATGGAATTGTAGCGCTACATACATACATACATACATAC |
| 8  | lacOMCS2 | CCTCGAGATTTGTAGCGCTACATACATACATACATACATAC |
| 9  | parB$p_{bg2b}$ | GATCGTTGCTGAGTCAGGCAG |
| 10 | parB$p_{bg2c}$ | GATCGTTGCTGAGTCAGGCAG |
| 11 | NulaciF | GGTCGCCGACGTAGACGACATTCG |
| 12 | PvalciR | GGCAGATCGATAAGCTGCAATTCG |
| 13 | onTRK2F | AGCGTCGACACATACGATGTTGCTGCGTTTCAGT |
| 14 | onTRK2R | CGGAATTCAATATGTGAAAGGTGTTGCTGAGT |
| 15 | oriTRK2F | CGGAATTCAATATGTGAAAGGTGTTGCTGAGT |
| 16 | oriTRK2R | CGGAATTCAATATGTGAAAGGTGTTGCTGAGT |
| 17 | NcoA | ACCATGGTCATG |

Restriction enzyme recognition sites are in bold, mutated nucleotides are indicated by lowercase, parS palindrome is underlined.

Primers used in site-directed mutagenesis

| No | Name | Sequence |
|----|------|----------|
| 18 | muttrf$p_{RK2}$ | GTTCTTGAGAAGAGAAGTCGTCGTTAGC |
| 19 | muttrf$p_{RK2}$ | CCAGTGTGTTGCTCAGTCCTTTC |
| 20 | xhoF8180 | CGGGCGTCGAGCTCTGAGACGTATCATCTGAC |
| 21 | xhoR8180 | CGATATGATGCTCGAGCCGACGGCGC |
| 22 | apaF9950 | CTCTCTGTTGCGGCGCCAAAGTTTTATCGTTGTTTCC |
| 23 | apaR9950 | GAAACCAAGCAATTTTTTGCAGCCGCAACAAAGC |
| 24 | Ppu618F | CGTGCAGAGGCGAGCCGCAGCGTTAAT |
| 25 | Ppu618R | GCCAAGCTTGGTCAGCATAGCTGCCAC |

Restriction enzyme recognition sites are in bold, mutated nucleotides are indicated by lowercase, parS palindrome is underlined.

Primers used for Real Time qPCR

| No | Name | Sequence |
|----|------|----------|
| 26 | galKF | ATGATCCTTTCTTGGACGAC |
| 27 | galKR | AGCAGCCTTTATCTCTGAGGC |
| 28 | trfAF | GTGAAGATCACCTACGCAG |
| 29 | trfAR | TGGCAAAGCTGCTGAAAGG |
| 30 | repBRA3F | CATCGAGAAGCAAAAGGCG |
| 31 | repBRA3R | CCAAGCTTGGTCAGCATAGCTGCCAC |
Standard plate test of plasmid stability

Cultures of hosts carrying various plasmids were grown in L broth with selective antibiotics at 37 °C or 28 °C. Plasmid content in the initial cultures was assessed by plating 100 μl of diluted cultures onto L agar to get approximately 100–200 colonies (usually 10^5-fold dilution) and then restreaking 100 colonies onto L agar with the selective antibiotic. Plasmid retention was expressed as the percentage of Cm^R colonies. The cultures were grown in L broth without antibiotics for up to 60 generations (diluted 10^5-fold at the start and every 20 generations) and plasmid retention was analyzed as described above. No IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the cultures with the exception of experiments with E. coli DH5α (pABB34) where the effect of IPTG concentration was analyzed.

Rapid plate screening for plasmid retention

Transformants were grown overnight and diluted as described above. Every 20 generations 100 μl of 10^5-fold diluted culture was plated on L agar (for plasmids with klcAp-xylE fusion) or L agar with X-gal (for plasmids with klcAp-lacZ fusion). Colonies formed by bacteria with plasmids carrying the klcAp-xylE fusion become yellow after being sprayed with 10 mM catechol solution. Plasmid retention was calculated as the percentage of yellow colonies or blue colonies on X-gal for plasmids with klcAp-lacZ fusion.

Catechol 2,3-dioxygenase activity assay

The level of xylE expression from klcAp was determined by an enzymatic assay in extracts from logarithmically growing cultures of E. coli DH5α transformants using a standard method [27]. Protein concentration was assayed by the Bradford method [54]. One unit of catechol 2,3-dioxygenase activity is defined as the amount of enzyme necessary to convert 1 μmol of catechol to the yellow hydroxymuconic semialdehyde in 1 min under standard conditions.

Simplified XylE activity assay was applied to estimate plasmid stability in liquid cultures. Overnight cultures of E. coli DH5α transformants carrying test plasmids with klcAp-xylE (grown without selection for 20 or 40 generations) were diluted 100-fold, the cell densities were estimated by OD_{600} 1/10 volume of 10 mM catechol was added and absorbance at 405 nm was measured after 5 min. The OD_{405}/OD_{600} ratio clearly differentiated strains with plasmids of various stability. To quantify plasmid retention a high-throughput procedure with 100-well plates (Labsystems Honeycomb 2 plate) and a Bioscreen C Microbiology Reader Analyser (Labsystems) was used. Details are described in the Results section.

Determination of plasmid copy number by quantitative real-time PCR (qPCR)

The copy number of pRK415 and its derivatives was measured by qPCR using the SYBR Green JumpStart Taq ReadyMix kit (Sigma). The single-copy galK, gene from E. coli chromosome, was used as the chromosomal reference gene (primers #26 and #27) for all strains. The trfA gene of RK2 was used as the plasmid reference gene (primers #28 and #29) for pRK415 derivatives and pMPB9.90 (araBADp-trfA_RK2) whereas repB gene was amplified for plasmid RA3 (primers #30 and #31) (Table 2). Total DNA was purified from 4 ml of stationary-phase cultures using Genomic Mini purification kit (A&A Biotechnology), treated with an appropriate restriction enzyme to linearize the plasmid DNA and to fragment chromosomal DNA and then used as a template in qPCR. Plasmid copy number (PCN) was calculated relatively to the chromosomal marker on the basis of at least three biological replicates with three technical replicates per strain and average results with standard deviation are reported. The amplification, detection and analysis were carried out in the Laboratory of Genetic Modification Analyses of IBB PAS on an Applied Biosystems 7500 apparatus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AAB and GJB designed study. AAB, KG and GJB wrote the manuscript. AAB, AK, EL, JG participated in vectors construction. AAB, KG, AK, EL, AM and JG conducted plasmid stability tests. KG performed high-throughput quantification of plasmid retention. AM prepared templates for plasmid copy number determination. AAB, KG, AK, EL, JG, AM and GJB participated in data analysis. All authors read and approved the final manuscript.

Authors' information

1 Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Department of Microbial Biochemistry, 02-106 Warsaw, Pawinskiego 5A, Poland;
2 Present address: Warsaw University of Technology, Faculty of Chemistry, Institute of Biotechnology, 00-664 Warsaw, Poland;
3 Present address: Warsaw University of Life Sciences, Faculty of Human Nutrition and Consumer Sciences, Laboratory of Food Chemistry, 02-776 Warsaw, Poland;
4 Present address: Pulawska 255A/4, 02-740 Warsaw, Poland.

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Author details

1 Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland. 2 Present address: Faculty of Chemistry, Institute of Biotechnology, Warsaw University of Technology, Warsaw, Poland. 3 Present address: Faculty of Human Nutrition and Consumer Sciences, Laboratory of Food Chemistry, Warsaw University of Life Sciences, Warsaw, Poland. 4 Present address: Pulawska 255A/4, 02-740 Warsaw, Poland.

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