RESEARCH ARTICLE

Plasmid-Encoded RepA Proteins Specifically Autorepress Individual \textit{repABC} Operons in the Multipartite \textit{Rhizobium leguminosarum} bv. \textit{trifolii} Genome

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Abstract

Rhizobia commonly have very complex genomes with a chromosome and several large plasmids that possess genes belonging to the \textit{repABC} family. RepA and RepB are members of the ParA and ParB families of partitioning proteins, respectively, whereas RepC is crucial for plasmid replication. In the \textit{repABC} replicons, partitioning and replication functions are transcriptionally linked resulting in complex regulation of \textit{rep} gene expression. The genome of \textit{R. leguminosarum} bv. \textit{trifolii} TA1 (RtTA1) consists of a chromosome and four plasmids (pRleTA1a-d), equipped with functional \textit{repABC} genes. In this work, the regulation of transcription of the individual \textit{repABC} cassettes of the four RtTA1 plasmids was studied. The involvement of the RepA and RepB as well as \textit{parS}-like centromere sites in this process was depicted, demonstrating some dissimilarity in expression of respective \textit{rep} regions. RtTA1 \textit{repABC} genes of individual plasmids formed operons, which were negatively regulated by RepA and RepB. Individual RepA were able to bind to DNA without added nucleotides, but in the presence of ADP, bound specifically to their own operator sequences containing imperfect palindromes, and caused operon autorepression, whereas the addition of ATP stimulated non-specific binding of RepA to DNA. The RepA proteins were able to dimerize/oligomerize: in general dimers formed independently of ATP or ADP, although ATP diminished the concentration of oligomers that were produced. By the comprehensive approach focusing on a set of plasmids instead of individual replicons, the work highlighted subtle differences between the organization and regulation of particular \textit{rep} operons as well as the structures and specificity of RepA proteins, which contribute to the fine-tuned coexistence of several replicons with similar \textit{repABC} cassettes in the complex bacterial genome.
Introduction

Alphaproteobacteria commonly have very complex genomes with a chromosome and plasmids, which may significantly vary in the size and content. Bacteria of the *Rhizobiaceae* family have numerous, large plasmids, all belonging to the *repABC* family [1]. An important feature of rhizobial plasmids is their low-copy number in the cell and such plasmids rely on partitioning systems (*par*), distributing newly replicated plasmids to daughter cells [2–4]. Partitioning systems have been well described for several enterobacterial strains harbouring single plasmids like R or F, and episomal prophages like P1 [5–7]. Most of the low-copy number plasmids possess a Type I segregation system with Walker-type ATPase (MinD/ParA superfamily) [1]. Sub-type Ia comprises large ATPases with a DNA-binding domain in their N-terminus [8, 9].

The *repABC* plasmid family has several characteristics in common. Most notably, all elements necessary for active segregation and replication, as well as those responsible for incompatibility, are located in the same operon [10, 11], and the genetic organization of the *repABC* cassette is well conserved: *repA* is upstream of *repB*, and both precede *repC* [1, 11–13]. Despite their apparent structural homogeneity, *repABC* operons have diverse DNA sequences. They vary with respect to the presence of peptide-encoding minigenes, the numbers and class of the regulatory elements involved in operon transcription, and the numbers and positions of centromeric *parS* sequences [1, 14]. The structural diversity of *repABC* operons resulting from their complex and independent evolutionary history [15] may affect the regulation and functioning of particular replication systems.

RepA and RepB are members of the ParA and ParB families of partitioning proteins, respectively [6]. The size and sequence similarities of the RepA and RepB suggest that the partition systems of most of the rhizobial *repABC* replicons are Type Ia [1]. RepA and RepB play dual roles in plasmid maintenance: in conjunction with *parS* centromere-like sites, they participate in partitioning and in the negative transcriptional regulation of their own operons [16–20]. RepA-ADP can bind specifically to the operator preceding the *repABC* operon of p42d (formally known as pRetCFN42d) for repression of transcription [21]. RepC protein is crucial for plasmid replication and does not show similarities with members of other protein families. Its expression, down-regulated by small antisense RNA, encoded in the *repB-repC* intergenic region [22–27], is required and sufficient for replication of a plasmid [10, 28], which indicates that the origin of replication lies within the *repC* gene [28, 29]. Comprehensive analyses of the mechanisms of replication, segregation, and transcriptional regulation of several *repABC* operons coexisting in one cell are still scarce especially for the rhizobial model.

The genome of *R. leguminosarum* bv. *trifolii* TA1 (RtTA1) consists of five replicons: the chromosome and four plasmids pRleTA1a, pRleTA1b, pRleTA1c and pRleTA1d (497 kb, 598 kb, 646 kb and 798 kb, respectively), with the smallest, pRleTA1a, recognized as a symbiotic plasmid (pSym) carrying genes responsible for symbiosis with clover [30]. Recently, we have shown that all RtTA1 plasmids are equipped with functional *repABC* genes [31]. The complex evolutionary history and structural diversity of the RtTA1 *repABC* cassettes was demonstrated. They differed in the numbers and positions of *parS* and incompatibility elements (*inc*α) located in the *repB-repC* intergenic regions, and this was especially apparent in the case of pSym. Incompatibility assays with recombinant constructs containing *parS* or *inc*α demonstrated that RtTA1 plasmids belong to different incompatibility groups and were acquired by separate events of lateral transfer, as shown by phylogenetic analyses. pRleTA1a (pSym) was probably the evolutionary newest plasmid of this strain [31].

The aim of this study was to provide molecular characterization of partition proteins of four plasmids coexisting in one cell. Special attention was paid to the domain structure, and specific DNA-binding properties of RepA proteins of individual RtTA1 plasmids. The regulation of
transcription of four repABC cassettes was studied in detail and the involvement of the RepA and RepB as well as parS-like elements in this process was depicted, demonstrating some dissimilarity in the expression of the rep cassettes of respective plasmids. Operators constituting RepA binding sites of the individual RtTA1 plasmids were mapped. Moreover, the oligomerization propensity of the individual RepA proteins was analysed and its role in both the plasmid partition and regulation of transcription of repABC genes was discussed. To our knowledge, this is the first successful attempt at such a comprehensive approach focused on a set of plasmids instead of individual ones that sheds light on the problem of coexistence of several replicons equipped with similar repABC cassettes in the complex bacterial genome.

**Materials and Methods**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were cultured at 37°C or 30°C (*E. coli cya* strain DHM1) in LB medium [32]. *Rhizobium* and *Agrobacterium* strains were grown at 28°C in 79CA medium [33] or in M1 minimal medium [32]. Antibiotics, when used, were added at the following final concentrations (μg/ml): ampicillin, 100; kanamycin, 40 (25 for propagation of *E. coli M15[pREP4]* strain); rifampicin, 40; chloramphenicol, 30; tetracycline, 40; gentamicin, 10 for *Rhizobium*, 5 for *E. coli*.

**DNA manipulation techniques**

Standard techniques were employed for genomic DNA and plasmid isolation, PCR, molecular cloning, agarose gel electrophoresis and transformation [32]. Restriction and ligation reactions were conducted according to conditions specified by the manufacturer of the enzymes (Thermo Scientific). PCR was performed using high fidelity DNA polymerase (Sigma-Aldrich). The primers used in this work were obtained from Genomed (Warsaw, Poland) and are listed in S1 Table. Automatic sequencing was performed using BigDye Terminator Cycle Sequencing Kit and ABI PRISM 310 or Applied Biosystems 3500 Genetic Analyzers (Applied Biosystems).

**Construction of rep-lacZ transcriptional and translational fusions**

Transcriptional activity of *in silico* predicted promoters (Prep) of repABC cassettes of four RtTA1 plasmids was studied in a series of transcriptional fusions with promoterless lacZ gene in the pMPK reporter vector, which is pMP220 derivative [34] constructed in this work (Table 1). To construct these plasmids appropriate DNA fragments were PCR amplified using an upstream primer for the putative promoter region equipped with KpnI restriction site and the reverse primer, annealing within the coding sequence of relevant repA gene equipped with XbaI site, and cloned into pMPK. The recombinant plasmids were transferred into *A. tumefaciens* GMI9023 (*Atu*) by electrotransformation and the promoter activities were determined by measurement of the β-galactosidase activity. To define if repABC genes form an operon, the PCR amplified DNA fragments comprising Prep, repA, repB and 5’ end of repC were transcriptionally fused with lacZ in KpnI-XbaI sites of pMPK, and reporter activity was measured in *Atu* to avoid presumable incompatibility of such constructs in RtTA1. To reveal potential autoregulation of repABC operons and involvement of centromere-like parS sequences in this process, a set of deletion derivatives of particular repABC operons transcriptionally fused with lacZ in pMPK were constructed. The constructs comprised either Prep, repA and repB (with or without respective parS elements) or only Prep and repA. The respective DNA fragments were PCR amplified, cloned into KpnI-XbaI sites of pMPK and β-galactosidase activity was assayed in *Atu*. To test the potential ability of RepA and RepB to exert negative autoregulation on the
Table 1. Strains and plasmids used in this study.

| Strains and plasmids | Description | Source or reference |
|----------------------|-------------|---------------------|
| **E. coli**          |             |                     |
| DH5α                 | supE44 ΔlacU169 (880 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | [32] |
| JM101                | supE thi-1 Δ(lac-proAB) F' [traD36 proAB+ lacIq lacZΔM15] | [32] |
| M15[pREP4]           | NalR, StrR, RifR, Thi, Lac, AraR, GalR, MitR, F', RecA', UvrA', LonR, carrying repressor vector pREP4 | Qiagen |
| DHM1                 | F', cya-854, recA1, endA1, gyrA96 (NalR), thi1, hsdR17, spoT1, rfbD1, glnV44(AS) | [42] |
| Rosetta 2(DE3)       | FompT hsdSde(t6 ma) gal dcm (DE3) pLysSpRARE2 (Cm') | Novagen |
| **Rhizobium**        |             |                     |
| TA1                  | R. leguminosarum bv. trifolii, wild type, Str', Rif' | [50] |
| **Agrobacterium**    |             |                     |
| GM19023              | A. tumefaciens Str', Rif' cured of pAt and pTi | [51] |
| **Plasmids**         |             |                     |
| pBBR1MCS-2           | mob, Km' | [35] |
| pBBR1MCS-5           | mob, Gm' | [35] |
| pOE-30               | ori ColE1, Amp', expression vector | Qiagen |
| pET-32a(+)           | ori pBR322, Amp', trxA, expression vector | Novagen |
| pMP220               | IncP, mob, promoterless lacZ, Tet' | [34] |
| pMPK                 | pMP220 derivative with Kan' cassette from pBBR1MCS-2 inserted in EcoRI-KpnI sites | This work |
| **pMPK derivatives—lacZ transcriptional fusions** |             |                     |
| repABC of pRleTA1a   |             |                     |
| pMa1-pMa3            | pMPK with 576 bp, 269 bp, 198 bp KpnI-XbaI fragments comprising respectively 433 bp, 126 bp and 55 bp upstream of repA and 143 bp of repA | This work |
| pMa/ABC2             | pMPK with 2956 bp KpnI-XbaI fragment comprising 269 bp upstream of repA, repA, repB and 277 bp of repC | This work |
| pMa/AB2              | pMPK with 2371 bp KpnI-XbaI fragment comprising 269 bp upstream of repA, repA, repB and 1 bp downstream of repB | This work |
| pMa/A2               | pMPK with 1543 bp KpnI-XbaI fragment comprising 269 bp upstream of repA, repA and 203 bp of the coding region of repB | This work |
| repABC of pRleTA1b   |             |                     |
| pMb1-b2              | pMPK with 361 bp and 195 bp KpnI-XbaI fragments comprising respectively 262 bp and 96 bp upstream of repA and 99 bp of repA | This work |
| pMb3                 | pMPK with 255 bp KpnI-XbaI fragment comprising 37 bp upstream of repA and 218 bp of repA | This work |
| pMb/ABC2             | pMPK with 2677 bp KpnI-XbaI fragment comprising 96 bp upstream of repA, repA, repB and 229 bp of repC, devoid of parS1 element | This work |
| pMb/AB1              | pMPK with 2472 bp KpnI-XbaI fragment comprising 262 bp upstream of repA, repA, repB and 13 bp downstream of repB | This work |
| pMb/AB2              | pMPK with 2306 bp KpnI-XbaI fragment comprising 96 bp upstream of repA, repA, repB and 13 bp downstream of repB, devoid of parS1 element | This work |
| pMb/A2               | pMPK with 1318 bp KpnI-XbaI fragment comprising 96 bp upstream of repA, repA and 20 bp of repB | This work |
| repABC of pRleTA1c   |             |                     |
| pMc1-c2              | pMPK with 379 bp and 200 bp KpnI-XbaI fragment comprising respectively 286 bp and 107 bp upstream of repA and 93 bp of repA | This work |
| pMc3                 | pMPK with 301 bp KpnI-XbaI fragment comprising respectively 40 bp upstream of repA and 261 bp of repA | This work |
| pMc/ABC2             | pMPK with 2733 bp KpnI-XbaI fragment comprising 107 bp upstream of repA, repA, repB and 223 bp of repC | This work |
| pMc/AB1              | pMPK with 2539 bp KpnI-XbaI fragment comprising 286 bp upstream of repA, repA, repB and 49 bp downstream of repB | This work |
| pMc/AB2              | pMPK with 2509 bp KpnI-XbaI fragment comprising 86 bp upstream of repA, repA, repB and 19 bp downstream of repB, devoid of parS2 element | This work |
| pMc/AB3              | pMPK with 2360 bp KpnI-XbaI fragment comprising 107 bp upstream of repA, repA, repB and 49 bp downstream of repB, devoid of parS1 element | This work |

(Continued)
Table 1. (Continued)

| Strains and plasmids | Description | Source or reference |
|----------------------|-------------|---------------------|
| pMc/AB4              | pMPK with 2330 bp KpnI-XbaI fragment comprising 107 bp upstream of repA, repA, repB and 19 bp downstream of repB, devoid of both parS1 and parS2 elements | This work |
| pMc/A2               | pMPK with 1483 bp KpnI-XbaI fragment comprising 107 bp upstream of repA, repA and 162 bp of repB | This work |
| repABC of pRleTA1d    | pMPK with 540 bp, 316 bp, 272 bp, 242 bp and 217 bp KpnI-XbaI fragments comprising respectively 334 bp, 110 bp, 66 bp, 36 bp and 11 bp upstream of repA and 206 bp of repA | This work |
| pMd1-d5              | pMPK with 2832 bp KpnI-XbaI fragment comprising 110 bp upstream of repA, repA, repB and 369 bp of repC | This work |
| pMd/ABC2             | pMPK with 2339 bp KpnI-XbaI fragment comprising 110 bp upstream of repA, repA, repB and 30 bp downstream of repB | This work |
| pTd/AB2              | pMPK with 1488 bp KpnI-XbaI fragment comprising 110 bp upstream of repA, repA and 181 bp of repB | This work |
| pBBR1MCS-5 derivatives | pBBR1MCS-5 with 2371 bp KpnI-XbaI fragment of pRleTA1a comprising 269 bp upstream of repA, repA, repB and 1 bp downstream of repB | This work |
| pBa/A2               | pBBR1MCS-5 with 1543 bp KpnI-XbaI fragment of pRleTA1a comprising 269 bp upstream of repA, repA and 203 bp of repB | This work |
| pBa/A2               | pBBR1MCS-5 with 2472 bp KpnI-XbaI fragment of pRleTA1b containing 262 bp upstream of repA, repA, repB and 13 bp downstream of repB | This work |
| pBa/A2               | pBBR1MCS-5 with 2306 bp KpnI-XbaI fragment of pRleTA1b containing 96 bp upstream of repA, repA, repB and 13 bp downstream of repB | This work |
| pBa/A2               | pBBR1MCS-5 with 1484 bp KpnI-XbaI fragment of pRleTA1b containing 262 bp upstream of repA, repA and 20 bp of the coding region of repB | This work |
| pBa/B                | pBBR1MCS-5 with 1318 bp KpnI-XbaI fragment of pRleTA1b containing 96 bp upstream of repA, repA and 20 bp of the coding region of repB | This work |
| pBa/B                | pBBR1MCS-5 with pQb/B inserted in XbaI site | This work |
| constructs in expression plasmids | | |
| pQ-A/a-d             | pQE-30 with 1215 bp BamHI-KpnI and 1203 bp, 1215 bp and 1191 bp BamHI-HindIII fragments containing respective pRleTA1a-d repA coding sequences without start codon | This work |
| pQa-b/B, pQd/B       | pQE-30 with 1026 bp, 993 bp and 999 bp BamHI-HindIII fragments containing respective repB coding sequence of pRleTA1a-b and pRleTA1d without start codon | This work |
| pETc/B               | pET-32a(+) with 984 bp BamHI-HindIII fragment containing repB coding sequence of pRleTA1c without start codon | This work |
| BACTH system plasmids and constructs carrying repA and repB of pRleTA1b | | |
| pUT18                | Two-hybrid plasmid for cyaAT18 fusion construction, Amp' | [42] |
| pUT18C               | Two-hybrid plasmid for cyaAT18 fusion construction, Amp' | [42] |
| pKT25                | Two-hybrid plasmid for cyaAT25 fusion construction, Km' | [42] |
| pKNT25               | Two-hybrid plasmid for cyaAT25 fusion construction, Km' | [42] |
| pUT18C-zip           | Two-hybrid control plasmid | [42] |
| pKT25-zip            | Two-hybrid control plasmid | [42] |
| RepA/RepB-T18        | pUT18 carrying 1200 bp and 990 bp XbaI-KpnI fragments of repA/repB coding sequences devoid of start and stop codons | This work |
| T18-RepA/RepB        | pUT18C carrying 1203 bp and 994 bp XbaI-KpnI fragments of repA/repB coding sequences devoid of start and stop codons | This work |
| T25-RepA/RepB        | pKT25 carrying 1204 bp and 994 bp XbaI-KpnI fragments of repA/repB coding sequences devoid of start and stop codons | This work |
| RepA/RepB-T25        | pKNT25 carrying 1203 bp and 990 bp XbaI-KpnI fragments of repA/repB coding sequences devoid of start and stop codons | This work |
| T25-RepA-79401       | pKT25 carrying 993 bp XbaI-KpnI fragment of truncated repA, encoding the 79–401 amino acid residues of RepA | This work |

Abbreviations: Str', streptomycin resistance; Rif', rifampicin resistance; Km', kanamycin resistance; Gm', gentamicin resistance; Amp', ampicillin resistance; Tet', tetracycline resistance, Cm', chloramphenicol resistance.

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The repA and repAB genes of plasmids pRleTA1b and pRleTA1a were cloned into *KpnI*-*XbaI* sites of pBBR1MCS-5 [35]. To reveal the contribution of RepB alone on the repABC/b expression, *BamHI*-*HindIII* fragment containing repB coding sequence of pRleTA1b was cloned into pQE-30, and the recombinant plasmid was subsequently digested with *XbaI* and recloned into pBBR1MCS-5, resulting in the pBb/B. All constructs were verified by sequencing.

### β-Galactosidase activity measurements

The *A. tumefaciens* strains carrying the *lacZ* transcriptional fusions in pMPK were grown overnight in 79CA medium in the presence of kanamycin. The cultures were diluted in fresh M1 medium supplemented with a vitamin mixture according to Brown and Dilworth [36] and grown to mid-log phase. The *E. coli* strains were grown and diluted in LB medium. The level of *lacZ* expression was determined in Miller units, by assaying β-galactosidase activity with the ONPG (2-Nitrophenyl-β-D-galactopyranoside, MP Biomedicals) as a substrate, as described by Miller [37].

### Overproduction and purification of recombinant RepA proteins

For the expression and purification of recombinant RepA/a-d proteins with N-terminal His<sub>6</sub>-tag the relevant *repA* genes were PCR amplified using appropriate primer pairs (S1 Table), high fidelity DNA polymerase and RrTA1 genomic DNA as a template. Amplification products were cloned into *BamHI* and *HindIII* sites or *BamHI* and *KpnI* sites of pQE-30 vector (Qiagen) resulting in pQ-A/b-d and pQ-A/a recombinant plasmids, respectively. The following *E. coli* strains were used for propagation of the expression constructs: M15[pREP4] for pQ-A/a, pQ-A/b and pQ-A/d plasmids or JM101 for pQ-A/c. Strains carrying recombinant plasmids expressing RepA were grown at 37°C in LB broth containing appropriate antibiotics. IPTG (isopropyl-β-D-thiogalactopyranoside, Thermo Scientific) was then added to a final concentration of 0.1 mM when the cultures reached an OD<sub>600</sub> of 0.6 (pQ-A/a, pQ-A/d) or 1.0 (pQ-A/b, pQ-A/c), and incubation was continued for 5 hours or overnight, respectively. The cells were then chilled on ice, harvested by centrifugation, resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) supplemented with lysozyme to 1 mg/ml (MP Biomedicals) and protease inhibitor cocktail (Sigma-Aldrich), and incubated on ice for 30 min. The suspensions containing His<sub>6</sub>-RepA/a were additionally treated with DNase I (5 μg/ml, MP Biomedicals) on ice for 10 min. The cells were then disrupted using the FRENCH Pressure Cell Press (Thermo Scientific) and centrifuged at 10,000 × g for 20 min at 4°C to separate the soluble and cellular debris fractions. The supernatants were then subjected to His<sub>6</sub>-tagged protein purification via Co<sup>2+</sup> affinity chromatography using TALON Metal Affinity Resin (Clontech Laboratories, Inc.). The samples were mixed with affinity resin, gently agitated on ice in order to allow the His<sub>6</sub>-tagged proteins to bind the resin, then washed twice with wash buffer I (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and then once with wash buffer II (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). In the case of His<sub>6</sub>-RepA/a, extra-washing step with wash buffer III (50 mM phosphate, 1 M NaCl, 10 mM imidazole, pH 8.0) just before rinsing with wash buffer II was necessary, in order to get rid of DNA bound to proteins. Next, elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0) was applied and the fractions containing His<sub>6</sub>-RepA proteins were pooled and stored in 4°C. Samples were analyzed by SDS-PAGE, Western immunoblotting with anti-His<sub>6</sub> antibodies (Roche) and quantitated using Qubit Fluorometer (Invitrogen).
Overproduction and purification of recombinant RepB proteins

The relevant repB genes were PCR amplified and cloned into BamHI and HindIII sites of pQE-30 vector (Qiagen) (pQa/B, pQb/B, and pQd/B plasmids) or pET-32a(+) (Novagen) (pETc/B plasmid). E. coli M15[pREP4] and Rosetta 2(DE3)pLysS strains were used for propagation of the pQE-30-based and pET-32a(+) based expression constructs, respectively. Strains carrying recombinant plasmids expressing RepB were grown at 37°C in LB broth containing appropriate antibiotics. IPTG was then added to a final concentration of 0.5 mM when the cultures reached an OD600 of 0.6 and incubation was continued for 5 hours. Further steps in isolation and purification of RepB recombinant proteins were performed as described above for His6-RepA proteins.

Electrophoretic mobility shift assay (EMSA)

Non-radioactive electrophoretic mobility shift assay was used for analysis of recombinant RepA proteins binding with DNA. The target DNA fragments subjected to EMSA were PCR amplified with appropriate primers (S1 Table), high fidelity DNA polymerase and RtTA1 genomic DNA as a template. The target fragments encompassed the promoter and operator regions of the individual RtTA1 repABC cassettes, as well as their 5’ and 3’ sequential deletions of various length. The 148 bp non-specific control PCR product comprising fragment of the Km’ gene of the pBBR1MCS-2 [35] was employed in these experiments. DNA binding reaction was performed at 28°C for 30 min by incubating 5–25 ng of target DNA and 5–25 ng control DNA with 10–100 pmol of purified recombinant RepA proteins in binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 5 mM DTT, 100 μg bovine serum albumin and 5% [v/v] glycerol). The final volume of the reaction mixture was 20 μl. The ATP or ADP was present in the binding reaction when necessary in final concentration of 0.1, 1 or 2 mM. After incubation, 1 μl of loading dye (0.03% [w/v] xylene cyanol, 0.03% [v/v] bromophenol blue, 60% [v/v] glycerol) was added, and the samples were immediately loaded and separated by electrophoresis at room temperature on native 10% polyacrylamide gel (79:1) in TB buffer supplemented with 1 mM MgCl2. The gels were then stained with SYBR Green (Sigma-Aldrich) or ethidium bromide (Sigma-Aldrich) and exposed to UV.

Cross-linking experiments

The His6-RepA proteins (0.1–0.2 nM) stored in elution buffer were subjected to cross-linking experiments. DMP (dimethyl pimelimidate, Sigma-Aldrich) was added to the reactions at final concentrations of 0.5 mM, 1 mM, 10 mM and 25 mM. The final reactions volume was between 15 and 25 μl. The cross-linking reactions were incubated for 1 h at 28°C and then stopped by the addition of 1 μl of 0.5 M Tris-HCl (pH 6.8) followed by addition of 5× SDS loading buffer. The samples were heated at 95°C for 5 min and analyzed by SDS-PAGE followed by Western analysis with anti-His6 antibodies (Roche). In the time course experiment the 10 mM DMP concentration was fixed and the protein samples were incubated at 28°C from 30 s to 120 min. 2 mM ATP or ADP and 10–50 ng target Op DNA fragment or non-specific DNA competitor was added when needed.

Bacterial Two-Hybrid (BACTH) complementation assays

To construct the recombinant plasmids used in the BACTH complementation assays, the genes coding for the RepA/b, RepB/b proteins or RepA/b subdomain were PCR amplified, using appropriate primers listed in S1 Table and the RtTA1 genomic DNA as a template. Amplified DNA fragments were digested with appropriate restriction enzymes and subcloned...
into the corresponding sites of the ’bait’/’prey' vectors: pUT18, pUT18C, pKT25 and pKNT25, in DH5α strain. The resulting recombinant plasmids expressed hybrid proteins in which the polypeptides of interest were fused to the N-terminus of T18 and T25 fragments of adenylate cyclase in pUT18 and pKNT25 or to the C-terminus of T25 and T18 fragments in pKT25 and pUT18C, respectively. DNA sequences of the cloned genes in all recombinant plasmids were verified by sequencing. Next, E. coli DHM1, which carries a 200-bp deletion within the cya gene, was sequentially transformed with all the combinations of recombinant BACTH plasmids. Initial scoring of potential interactions was achieved on the LB plates containing ampicillin, kanamycin, X-Gal (40 μg/ml) and IPTG (0.5 mM), which were incubated at 30°C for 48 h. For a quantitative measurement, bacteria were grown in LB broth in the presence of 0.5 mM IPTG and appropriate antibiotics at 30°C for 14 to 16 h. Measurement of β-galactosidase activity was performed according to a procedure described by Miller [37]. pKT25-zip and pUT18C-zip derivatives of ’bait’/’prey' vectors, carrying gene fragments encoding GCN4 leucine zipper motifs, and empty ’bait’/’prey' vectors pKT25, pKNT25, pUT18C and pUT18 were used as the positive and negative control, respectively. For reproducible and robust results, only freshly transformed colonies were used.

Bioinformatics tools

Sequence data were analyzed with Lasergene analysis software (DNASTAR, Inc.). The promoter regions were analyzed using the DBGP server at University of California, Berkeley [38] and Promoter 2.0 algorithm [39]. For protein secondary structure analyses the PSIPRED prediction method was used [40]. Putative helix-turn-helix (HTH) motifs were predicted using GYM 2.0 [41].

Results

repABC genes of the individual plasmids of RtTA1 form operons negatively regulated by RepA and RepB

In silico promoter prediction suggested operon organization of all RtTA1 repABC regions, with the putative promoter located upstream of the repA genes [31]. Moreover, in the putative promoter regions of all RtTA1 rep cassettes, operator-like palindromes were identified suggesting complex regulation of repABC expression. To study the transcription of the repABC genes of individual RtTA1 plasmids and regulation of the putative repABC operons with special attention to the contribution of RepA in this process, series of rep-lacZ transcriptional fusions were constructed and analysed (Fig 1).

Mapping of the promoters of repABC cassettes. First, promoters of individual RtTA1 repABC cassettes were mapped. DNA fragments comprising regions upstream of particular repA genes sequentially shortened at the 5’-end were PCR amplified and cloned into pMPK, creating a set of rep-lacZ transcriptional fusions (Fig 1A–1D), which were introduced into Atu. The reporter activities differed in fusions comprising promoters of particular repABC cassettes. The highest relative level of β-galactosidase was estimated for the repABC promoter of pRleTA1a (Fig 1A), while the LacZ activities of other rep regions were almost twofold lower (Fig 1A–1D). The LacZ activity of the pMa1-a2 was high, while in the case of the pMa3 it was drastically reduced. These results allowed mapping of the repA upstream elements essential for transcription initiation of the pRleTA1a rep cassette, which was located between -126-55 bp (Fig 1A). Noteworthy, pMa2 was able to express the reporter gene in E. coli, indicating that the RtTA1 rep promoters can be recognized by the E. coli transcription machinery. A sequence
Fig 1. Transcriptional activity of repABC operons of *Rhizobium leguminosarum* bv. *trifolii* TA1 plasmids: (A) pRleTA1a, (B) pRleTA1b, (C) pRleTA1c, and (D) pRleTA1d. Left panels show schematic genetic organization of the repABC cassettes. White arrows represent repA, repB and repC genes, and white broken arrows depict repA genes. Black dots show the position of parS-sites. The identified promoters of repABC operons were marked as grey rectangular boxes. The respective DNA fragments necessary for particular promoter identification, operon structure assignment, as well as their sequential deletions used for operon regulation studies, which were cloned into pMPK reporter and pBBR1MCS-5 vectors, were shown as black lines with
positions indicated relative to repA start codon. The following system was applied for pMPK- and pBBR-1MCS5-based constructs nomenclature: e.g. in pMa/AB2 recombinant plasmid the first two letters (pM) mean the shortcut of vector name (pMPK in this case), the next small letter ‘a’ means that it is derivative of pRleTA1a repABC cassette, the capital letter A followed by slash means that the cloned fragment comprise entire repA gene and expressing RepA and RepB genes and fragment of repC fused with lacZ). Respectively, pBBA2 means the same fragment recloned to the pBBR-1MCS5. Right panels represent constructs the cloned fragments encompass entire repABC cassettes, which were located between -96-37 bp and downstream of -107 bp for pRleTA1b and pRleTA1c plasmids, respectively. Similarly to pMa2, pMb2 and pMc2 were able to express the reporter gene in E. coli, and again sequences with some similarity to the E. coli σ70 promoter with putative -35 and -10 consensus boxes were recognized in the fragments cloned into pMb2 [GTGTCA(N22)TATGTT] and pMc2 [TCGGCA(N18)CAAAAT].

Surprisingly, to map the region essential for transcription initiation of the pRleTA1d rep cassette, five lacZ transcriptional fusions were required. The β-galactosidase activity of four constructed plasmids denoted as pMd1-d4 comprising a sequentially shortened 5′-end of the hypothetical promoter was still high up to pMd5, in which the LacZ activity was substantially reduced (Fig 1D). In the repA upstream region of pRleTA1d, several strong promoters were predicted. pMd1-d4 were able to express the reporter gene in E. coli, and within the cloned fragments, sequences similar to the E. coli σ70 promoter consensus were recognized: in the pMd1 [CTGACG(N17)TAAATT] and pMd2 [TTGTAA(N20)TATAGT], and in the pMd3 and pMd4 [TTCAAA(N17)GAAAAA].

It was concluded that the repABC/d promoter was most probably located downstream of the -36 bp position.

Operon organization of repABC cassettes. The operon organization of the RlTA1 repABC cassettes was verified subsequently with another set of pMPK-based lacZ transcriptional fusions (Fig 1A–1D). The pMa-d/ABC2 plasmids with fragments comprising the promoters defined above, repA, repB, ctrRNA gene, and part of repC, to which promoterless lacZ was fused, were still able to express β-galactosidase. The level of LacZ activity was very low, however, it differed significantly (p < 0.001) from the control pMPK, indicating that the repABC cassettes constitute operons (Fig 1A–1D). Moreover, the low reporter activity observed in all the tested repABC-lacZ fusions strongly suggested that RepA and/or RepB participated in the autorepression of the operons.

The role of RepA and RepB in operon autorepression. To examine the role of RepA and RepB in autorepression, a series of plasmids, which contained the respective promoters and expressed either RepA alone or both RepA and RepB proteins were tested for the ability to repress the transcription of respective operons. In the pMPK-based constructs containing the repA gene and expressing RepA in cis from its own promoter (pMa-d/A2 series), the β-galactosidase activity was decreased by approximately 30% for the repABC/a cassette, 20% for repABC/c, and 50% for repABC/d. However, it was not affected in the case of repABC/b, compared to the LacZ activity of the pMa-c2 and pMd-4 plasmids (Fig 1A–1D). This diverse RepA effect on the transcriptional activity of the particular rep operons once again demonstrated some dissimilarity between the individual repABC cassettes. In turn, in the pMPK derivatives with cloned repAB genes (without the ctrRNA gene located downstream of the respective repB) and expressing RepA and RepB in cis from the promoter preceding repA (pMa-d/AB series),
the LacZ activity was significantly diminished in the case of all the repAB-lacZ fusions (Fig 1A–1D). We have concluded that RepA and RepB acting together repress the individual repABC operons, resulting in negative autoregulation. We have also tested the ability of the RepA and RepB proteins to exert negative autoregulation of the operon when provided in trans. The repA and repAB genes of two plasmids pRleTA1a and pRleTA1b were cloned into pBBR1MCS-5 (Fig 1A and 1B). Introduction of pBa/A2 or pBb/A2 plasmids that expressed RepA into Atu harbouring pMa2 or pMb2, respectively, moderately repressed the lacZ expression of the pMa2 fusion (the level of the reporter activity was comparable to that observed in pMa/A2) and had almost no effect on the reporter activity in pMb2 (as in the case of pMb/A2) (Fig 1A and 1B). However, the activity of β-galactosidase in the relevant Prep-lacZ fusions was substantially decreased when a second vector, expressing both RepA and RepB (pBa/AB2 or pBb/AB1 and pBb/AB2), was introduced into Atu with pMa2 or pMb2, respectively (Fig 1A and 1B). Concomitantly, when pBb/B (Fig 1B) expressing only RepB/b was introduced into Atu with pMb1 or pMb2, the activity of β-galactosidase of neither of these two lacZ fusions was affected. These results show that RepA is important for autorepression but strong repression of the individual RtTA1 repABC operons is possible only in the presence of both RepA and RepB proteins, which can be provided in trans, but not in the presence of RepB only.

The role of parS elements in repABC operon expression. To study the role of parS centromere-like elements in the RtTA1 repABC operon expression, the pMPK-based transcriptional fusions with fragments containing repAB genes of the pRleTA1b and pRleTA1c but deleted for respective parS sequences were examined (Fig 1B and 1C). In each of these repABC cassettes, two parS sites were previously found, which introduced in trans into the RtTA1 genome exerted incompatibility against respective parental plasmids [31]. In the lacZ fusions comprising promoters, repA, repB, and just one parS site irrespective of its location, i.e. within the repB coding sequence (pMb/AB2), upstream of repA (pMc/AB2), or downstream of repB (pMc/AB3), the β-galactosidase activity was low (Fig 1B and 1C). The LacZ activity measured in Atu cells bearing the pMb/AB1 or pMc/AB1 constructs with two parS elements was also low and comparable to the β-galactosidase activity of the construct with just one centromere-like element (pMb/AB2 and pMc/AB2 or pMc/AB3) (Fig 1B and 1C). On the other hand, lacZ expression in the pMc/AB4 deleted for both parS sites was only slightly elevated in relation to pMb/AB1, pMc/AB2, or pMc/AB3 (Fig 1C). It was concluded that presence of the parS element was not mandatory to achieve strong repression of the repABC operon, whereas RepA-RepB protein interaction is crucial for the regulation.

Summarizing, these results showed that the repABC genes of all the RtTA1 plasmids were organized as operons and were negatively regulated by cooperating RepA and RepB proteins. Despite the similarity in the genetic organization of the individual RtTA1 rep operons, they differed slightly with respect to the location of the individual promoter, their transcriptional activity that was twofold higher for pRleTA1a in comparison to other plasmids, and response to the RepA regulatory protein.

Individual RtTA1 RepA proteins specifically bind to the operator for autorepression of operon

As demonstrated above, individual RepA can, at least partially, repress its own transcription. In each of the four RtTA1 plasmids, a putative operator sequence (Op) was found upstream of the repA gene initial codon. The operons contained imperfect palindromes, which in the case of the repABC/b, repABC/c, and repABC/d cassettes partially overlapped the identified promoters (Fig 2A–2D).
Fig 2. Mapping of operator sequences of repABC operons of RtTA1 plasmids: (A) pRleTA1a, (B) pRleTA1b, (C) pRleTA1c, and (D) pRleTA1d. Left panels show schematic depictions of the region upstream of repA of repABC operons. The respective promoters (grey rectangle), operators—imperfect palindromes (black inverted triangles), parS elements (black dots) and repA genes (white broken arrow) were shown. The sequence of each palindrome is highlighted in yellow. Length and relative position of DNA fragments used in operator (Op) mapping were shown with respect to repA start codon. Right panels represent EMSA results with recombinant His6-RepA and respective DNA fragments (5–25 ng). Black triangles indicate the increasing concentration of particular His6-RepA protein (10 and 100 pmol) present in DNA binding reaction. Black arrows indicate the position of the retarded DNA bands.
In the N-terminal part of the individual RepA proteins, elongated α-helices and helix-turn-helix (HTH) DNA binding motifs were predicted (Fig 3). To study the binding of RepA proteins to putative Op DNA sequences, we undertook EMSA analyses. Particular RepA were overexpressed in *E. coli* as N-terminally His<sub>6</sub>-tagged recombinant proteins, purified by affinity chromatography under native conditions, and used in series of non-radioactive EMSA with PCR-amplified DNA fragments of various lengths, comprising putative Op sequences of individual *repABC* operons (Fig 2A–2D). Using this *in vitro* approach for all of the His<sub>6</sub>-RepA RTTA1 proteins, their ability for binding to their own Op element was demonstrated (Fig 2A–2D). Moreover, the length of the operator sequence necessary for respective RepA binding was mapped and ranged from -55 to -6 bp for *repABC/a*, downstream of -96 bp for *repABC/b*, downstream of -40 bp for *repABC/c*, and downstream of -66 for *repABC/d* relative to the repA ATG codon (Fig 2A–2D). Noteworthy, no cross reactivity between individual RepA proteins and Op elements from non-parental *repABC* cassettes was demonstrated (Fig 4). The RepA proteins bound to their own *repABC* operator sites in a very specific manner, which is likely to correlate with operon autorepression. The negative regulation of transcription of *RtTA1 rep* operons was strongly enhanced by RepB, as demonstrated above. We performed the EMSA with respective recombinant RepB proteins and DNA fragments denoted a5, b2, c3, and d3 (Fig 2), comprising the minimal-length DNA segments upstream of *repA* necessary for respective His<sub>6</sub>-RepA binding, but no shifted bands were observed in this assay. These results indicate that individual RepB proteins may exert their corepressor action by binding to corresponding RepA (described later) rather than directly to the Op region.

The role of ATP/ADP in the DNA binding specificity of RepA proteins was examined in the EMSA, in which two DNA fragments were used: a specific—including the respective Op sequence—and a non-specific competitor (an internal fragment of the Km<sup>+</sup> gene of the pBBR1MCS-2) (Fig 5). For each of the recombinant *RtTA1* His<sub>6</sub>-RepA proteins, the addition of ATP stimulated its non-specific DNA binding, while in the presence of ADP the individual

![Fig 3](image-url)
RepA bound specifically to Op sequences (Fig 5A–5D). These results show that, in the presence of ADP, individual RepA bind specifically to their own Op sequence for rep operon repression.

RepA proteins are able to self-associate in vitro in an ATP/ADP-dependent manner and differ in the oligomerization pattern

Cross-linking experiments with dimethyl pimelimidate (DMP) were performed to characterize the ability of RtTA1 RepA to oligomerize. When individual His6-RepA/a–d were treated with a solution with an increasing concentration of DMP, cross-linked dimeric species were unambiguously detected (Fig 6A–6D). At a higher DMP concentration, bands corresponding to multi-meric fractions were observed for all of the RepA proteins (Fig 6).

A time course experiment with a fixed concentration of DMP (10 mM) revealed that each of the tested RepA was initially fixed into covalently bound dimers, but yet the multimeric forms were observed very soon (Fig 7). Three of the tested His6-RepA proteins, namely RepA/a, RepA/c, and RepA/d displayed similar kinetics of the respective dimer/multimer formation: they required from 3 to 5 min to form dimers/multimers, while after 15 min the reaction reached a plateau (Fig 7A, 7C and 7D). On the contrary, the RepA/b protein formed dimers rapidly in the solution: after 30 s of incubation with 10 mM of DMP at 28°C; after another 30 s, multimers were visible and after 3 min the reaction reached a plateau (Fig 7B).

To gain further insight into the RepA protein oligomerization pattern, additional components such as ATP, ADP, and specific and non-specific DNA in various combinations were included in the cross-linking reactions (Fig 8). In general, the oligomerization pattern of the His6-RepA proteins seemed not to be influenced in vitro by the specific or non-specific DNA. On the other hand, when ATP was present in a cross-linking reaction, the bands corresponding to putative multimers were absent or weakly visible for most of the tested RepA and this phenomenon was independent of the kind of tested DNA (Fig 8A, 8B and 8D). In the case of the
RepA/c protein, the presence of ATP in the cross-linking reaction did not affect the multimer formation (Fig 8C). However, the addition of ATP slightly diminished the intensity of bands corresponding to high molecular weight multimers (Fig 8C).

These results showed that individual RepA proteins formed dimers and multimers in the solution. The RepA proteins differed slightly in the oligomerization pattern, which was not dependent on the presence of specific or non-specific DNA but was affected by ATP or ADP.
BACTH analysis of RepA and RepB interplays

The results presented above demonstrated the self-association ability of RepA as well as the possibility of reciprocal RepA and RepB interaction in the regulation of repABC operons. To validate these results, a bacterial two-hybrid system (BACTH) [42] was employed for examination of interplays between RepA and RepB. Since numerous recombinant plasmid constructs are usually required in two-hybrid based studies, only proteins originating from the pRleTA1b plasmid were subjected to the analysis. Both the respective repA and repB genes were cloned into pUT18, pUT18C, pKT25, and pKNT25 vectors to construct T18- or T25-gene fusions in various combinations (Fig 9A). Then, the E. coli DHM1 cya reporter strain was sequentially...
transformed with all plasmids expressing fusion proteins. Positive clones with blue colouring and reporter activity substantially higher than in the control, representing the interacting RepA-RepA proteins, were obtained for one of the tested combinations of fusion plasmids RepA-T18/T25-RepA, demonstrating the ability of RepA to form homooligomeric forms \textit{in vivo} (Fig 9B). To map the putative oligomerization domain in the N-terminal part of the RepA protein, a truncated version of RepA comprising 79–401 aa was prepared as a 'prey' and cotransformed with a plasmid expressing RepA as a 'bait' (Fig 9A). No positive blue clones were obtained for this combination of fusion plasmids, in contrast to the high \(\beta\)-galactosidase activity observed for intact RepA-RepA proteins (Fig 9B). Altogether, these results suggested the location of the oligomerization domain in the N-terminus of the RepA protein. In the case of the RepA-RepB interaction, a high level of \(\beta\)-galactosidase activity was obtained in one combination of fusion plasmids (RepB-T18/T25-RepA) (Fig 9C). For several other clones, the

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**Fig 7. Time course cross-linking analysis of His\textsubscript{6}-RepA/a-d.** His\textsubscript{6}-tagged RepA proteins (0.1–0.2 nM) were incubated with DMP concentration fixed at 10 mM, at 28°C, from 30 s to 120 min, separated on 10% SDS-PAGE and visualized by Western blot with anti-His\textsubscript{6} antibodies. The thin lines indicate the marker bands whose molecular masses are expressed in kDa. Arrows indicate different species formed by RepA proteins corresponding to monomers and dimers, while the position of multimers was marked with brackets.

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The reporter activity was higher than in the negative control but substantially lower than in positive clones in which RepA-RepA interaction was observed (Fig 9B and 9C). The results obtained confirm the capability of RepA and RepB proteins of interactions in vivo, and the low reporter activities may indicate that such interaction was weak under the experimental conditions applied.

Fig 8. Oligomerization pattern of His6-RepA/a-d in the presence of ADP/ATP, specific and nonspecific DNA. Western blots are showing the products of cross-linking of individual His6-tagged RepA proteins (0.1–0.2 nM), following 1 h incubation at 28°C with 10 mM DMP and additional compounds. ‘+’ means the presence of: ‘C’—nonspecific DNA competitor (10–50 ng); ‘Op’—specific operator DNA (10–50 ng); ADP (2 mM) or ATP (2 mM). The thin lines indicate the marker bands whose molecular masses are expressed in kDa. Arrows indicate different species formed by RepA proteins corresponding to monomers and dimers, while the position of multimers was marked with brackets.

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Fig 9. BACTH analysis of interactions of RepA and RepB proteins. (A) The schematic representation of full length RepA and RepB, which were translationally fused to N- and C-terminus of T18 and T25 functional domains of CyaA protein, as well as constructed ‘prey’ hybrid plasmid which comprised N-terminally truncated RepA fused to T25 domain. (B) RepA/RepA and (C) RepA/RepB interactions in BACTH analysis which were quantified by measuring of β-galactosidase activity (Miller units) in hybrid cotransformants.
Discussion

Autoregulation of partition (par) operons is a common theme in low-copy number plasmids. However, knowledge about transcriptional regulation is somewhat scarce for most of the rhizobial repABC systems and well developed only for two plasmids: A. tumefaciens pTiR10 and R. etli p42d [17–19, 21, 43]. The diversity of repABC cassettes related to the specific structural and transcriptional regulatory elements may substantially affect the regulation and functioning of particular plasmids in a multipartite genome. Our comprehensive approach focusing on the entire set of plasmids instead of just one led to the observation that the level of transcription of the individual RtTA1 rep operons varied: the reporter activity in the lacZ fusion comprising promoter of pRleTA1a (pSym) was almost twice as high as that in other RtTA1 rep regions. In our previous studies, we showed that horizontal acquisition was the main plausible contributor to the origin of RtTA1 plasmids and pSym could be the newest plasmid of this strain [31]. The presence of putative tra-trb genes upstream of pRleTA1a repABC, which may be related to the increased transcriptional activity of pRleTA1a rep promoter, is another example supporting the diversity of this rep region. The repABC operon of pTiR10 is functionally linked with trb-tra genes and contains four promoters, which respond to diverse environmental signals that modulate its transcription [18, 43]. However, in the pRleTA1a traI-repA intergenic sequence, no conserved motifs or segments such as tra-boxes were found, which could be indicative of conjugative properties of this plasmid [1, 18]. Similarly, the R. etli p42d symbiotic plasmid contains a traI pseudogene upstream of the repABC operon but without tra boxes [19], suggesting its inability to detect quorum-sensing signals.

The individual RtTA1 repABC operons were negatively regulated by cooperation of RepA and RepB. Only two rhizobial RepA proteins have earlier been shown to mediate autorepression: the RepA of pTiR10 and the RepA of p42d [18, 19]. Both these proteins were recognized as major cis-acting autorepressors of respective operons causing substantial reduction of respective promoter activity. In our studies, the constructs expressing solely RepA in cis from its own promoter resulted in up to 50% reduction of promoter activity and, in the case of the repABC/b operon, RepA alone did not affect the promoter activity. Our data strongly suggest that RepA is important for autorepression but the full repression of individual RtTA1 repABC operons is possible only in the presence of both of RepA and RepB, which can be provided in trans. This diverse RepA effect on the transcriptional activity of particular RtTA1 rep operons further underlines some functional dissimilarity between the individual repABC cassettes residing in one cell. Recently, Pérez-Oseguera and Cevallos [21] have shown that RepA alone repressed the transcription of the p42d repABC operon only marginally, while both RepA and RepB decreased its activity by approximately 50%.

It was shown for the p42d plasmid that the elements required for full repression of the repABC operon comprise RepA, RepB in conjunction with parS, and operator sequences [21]. Chai and Winans [17] postulate that RepB increases the affinity of RepA for the binding site at the repABC P4 promoter of pTiR10, but also indicate that removal of the RepB binding site (i.e. parS) enhances the operon transcription approximately fourfold. Our results allowed us to conclude that presence of the parS element is not mandatory to achieve strong repression of the repABC operon, whereas RepA-RepB protein interaction seems to be crucial for such
regulation. However, since both RepA and RepB are DNA-binding proteins, it is rather hard to define unambiguously the exact nature of the protein–DNA and protein–protein interactions in such a heterogeneous nucleoprotein complex required for rep operon regulation. The presented results strongly support the action of RepB as a corepressor and this result stays in agreement with the data provided for p42d [21]. Furthermore, the data discussed above clearly suggest RepA and RepB interplay. By means of BACTH, we have shown that RepA and RepB interact in vivo, confirming engagement of these proteins in transcription regulation of the repABC operon and presumably (by similarity with ParA/ParB) in plasmid partition.

What is especially important, we have demonstrated highly specific RepA binding to the Op and no cross reactivity between individual RepA and Op from non-parental repABC cassettes. Since RepA/RepB are capable of negative regulation of their own transcription and this process plays a crucial role in plasmid incompatibility [21], the specific interaction between RepA and the Op element seems to be one of the fundamental aspects of coexistence of several plasmids equipped with a similar replication system in one bacterial cell.

The DNA binding properties of ParA family members are known to be strongly dependent on whether the protein is bound with ATP or ADP [44]. In the presence of ATP, ParA is capable of binding to non-specific DNA. ParB stimulates the ParA ATPase activity leading to the accumulation of the ParA-ADP repressor form in which ParA binds exclusively to the parAB operator [3, 45]. In this regard, all RtTA1 RepA proteins behave similarly to ParA: individual RepA-ADP can bind specifically to their own Op sequence, while the addition of ATP stimulates their non-specific DNA binding. Similar results with respect to the ATP/ADP effect on RepA-Op binding have recently been described for p42d [21]. Oppositely, the RepA binding affinity to the Op sequence overlapping the repABC P4 promoter of pTiR10 was equally increased by adenosine di- and triphosphates. The presence of RepB clearly stimulated binding of RepA in both presence and absence of the nucleotide cofactor [18]. The ability of RepA to bind the non-specific DNA implicates its involvement in plasmid partition. A model was presented in which P1 plasmids dynamically associate with the bacterial nucleoid via ParA, positioning plasmids on the bacterial nucleoid, which serves as a matrix support for plasmid partition [3, 46, 47].

Undoubtedly, both ParA activities, i.e. par operon regulation and plasmid partition, are related to its ability to dimerize/oligomerize: the ParA filaments can push or pull replicated plasmids apart [48]. In our study, for most of RepA, the dimers were formed independently of ATP or ADP, but the presence of ATP stabilized dimers and/or diminished the amount of oligomers. Similar data have recently been obtained for RepA of p42d [21]. The result is somewhat puzzling in comparison with what is known about ParA. The ParA-ADP structure revealed that ADP binding locks the proteins in a specific dimer state and mediates folding of its C-terminal region important for operator binding [49]. In our studies, the self-association ability of RepA was confirmed in vivo using a bacterial two-hybrid system (BACTH). Using this approach, we have also demonstrated that the N-terminal part of RepA/b comprising 78 aa residues is crucial for protein oligomerization. The crystal structure of ParA showed that its N-terminus contained an elongated α-helix, which functions in dimerization, as well as a winged helix-turn-helix DNA binding motif. Such a region is typical for Type Ia NTPases and is not found e.g. in the Ib class [4, 49]. A similar secondary structure was also predicted for the RtTA1 RepA proteins. It has recently been demonstrated that a p42d RepA mutant with a deletion of nine amino acid residues partially overlapping the HTH motif was unable to repress the operon transcription either alone or in the presence of RepB, showing that this region was crucial in DNA binding [21].

A more general conclusion from the data discussed above is that a slightly different mode employed for regulation of particular rep cassettes is a result of fine differences related to
organization of promoter-operator regions, secondary structures of RepA proteins, and their oligomerization abilities. On the other hand, the several functionally conserved RepA interact in a very specific manner only with parental operons allowing coexistence in one bacterial cell of numerous plasmids possessing similar replication/partition systems. It should be strongly emphasized that all of these functionally important details could come to light by the comprehensive approach focusing on the entire set of plasmids, which we employed.

Supporting Information
S1 Table. Primers used in this study.

Author Contributions
Conceived and designed the experiments: KZ MM AM. Performed the experiments: KZ PK. Analyzed the data: KZ PK MM AS AM. Wrote the paper: KZ PK MM AS AM.

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