Master Thesis

Construction of new Expression Vectors for *R. eutropha* H16

Elisabeth Dornisch

Institute of Molecular Biotechnology

Graz University of Technology

Mag.rer.nat. Dr.rer.nat.Petra Köfing

Univ.-Prof. Dipl.-Ing. Dr.techn. Helmut Schwab

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Abstract

The gram-negative and strictly respiratory facultative lithoautotrophic bacterium *Ralstonia eutropha* H16 has attracted significant interest due to its ability to grow on H_2 and CO_2 as sole energy and carbon source in the past years. An effective regulatory system enables *R. eutropha* H16 to live under different habitant conditions and to switch between heterotrophic and autotrophic growth modes. This makes it a model organism for studying important control mechanisms during autotrophic CO_2 fixation, hydrogen oxidation and denitrification. Furthermore, the ability to grow to high cell densities (up to 200 g dry weight/L) during litoautotrophic or heterotrophic conditions has made *R. eutropha* H16 to a favored production organism with high biotechnological potential. Due to the lack of transport capacities for inducers like lactose or IPTG only rather complex inducible expression system for heterologous gene expression are reported.

In order to establish a simple expression system, which is applicable under various growth conditions, new expression vectors for *R. eutropha* H16 with different replication systems using the P_{Tac} as constitutive promoter, the kanamycin resistance gene as selective antibiotic marker and *egfp* as reporter gene to monitor protein expression, were designed. The main focus of this work was the comparison of different origins of replications and their effect on the protein expression, as well as the application in *R. eutropha* H16. A set of four new expression vectors containing different replication origins and a partitioning function were constructed and their stable maintenance in *R. eutropha* H16 was proved. Possible effects of the origins on the protein expression levels were compared by the determination of expressed eGFP, applying diverse detection methods like SDS-PAGE, Western Blot analysis and the measurement of fluorescing units.

Zusammenfassung

Das gram-negative und streng fakultativ lithoautotrophe Bakterium *Ralstonia eutropha* H16 hat in den letzten Jahren signifikant an Interesse gewonnen, da es mittels H_2 und CO_2 als einziger Energie bzw. Kohlenstoffquelle wachsen kann. Ein effektives Regulationssystem ermöglicht *R. eutropha* H16 das Wachstum unter verschiedenen Bedingungen, sowie den Wechsel zwischen heterotrophen und autotrophen Wachstumsmodi. Dadurch wurde es zu einem Modellorganismus für die Analyse von wichtigen Kontrollmechanismen bei der autotrophen CO_2-Fixierung, Wasserstoffoxidierung und Denitrifizierung. Die Fähigkeit während lithoautotrophen oder heteroautotrophen Bedingungen zu sehr hohen Zelldichten (bis zu 200 g Trockengewicht/L) anzuwachsen hat dazu beigetragen hat, dass aus *R. eutropha* H16 ein beliebter Produktionsorganismus mit hohem biotechnologischem Potential wurde. Wegen fehlender Transportsysteme für Laktose oder IPTG sind aber hauptsächlich komplexere induizierbare Expressionssysteme beschrieben.

Um ein einfacheres Expressionssystem für verschiedene Wachstumsbedingungen zu etablieren wurden neue Expressionsvektoren für *R. eutropha* H16 mit unterschiedlichen Replikationsursprüngen designt, sowie mit P_{Tac} als konstitutiven Promoter, dem Kanamycin-Resistenzgen als selektiven Antibiotikamarker und *egfp* als Reportergen zur Kontrolle der Proteinexpression. Im Hauptfokus der Arbeit stand der Vergleich möglicher Effekte von verschiedenen Replikationsurprüngen auf die Proteinexpression und die Ermittlung eines geeigneten Replikationssystems für *R. eutropha* H16. Mittels der unterschiedlichen Replikationsursprünge und einem Partitionierungssystem wurde ein Set von neuen Expressionsvektoren konstruiert, die in *R. eutropha* H16 stabil replizierten. Mögliche Effekte der Replikationssursprünge auf die Proteinexpressionslevel konnten durch die Bestimmung des exprimierten eGFPs verglichen werden. Hierbei wurden verschiedene Detektionsmethoden, wie SDS-PAGE, Western Blot und die Messung der Fluoreszenzeinheiten angewendet.

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List of abbreviations

APS	Ammoniumpersulfat
bp	Base pairs
BSA	Bovine serum albumin
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
FSB	Formamid sample buffer
FU	Fluorescing units
Gen	Gentamycin
Kan	Kanamycin
kDA	Kilo Dalton
KP_i	Potassium phosphate buffer
LB	Luria Broth
MSM	Mineral salt medium
NB	Nutrient broth
OD_{600}	Optical density at 600 nm
ONC	overnight culture
oNP	ortho-Nitrophenol
oNPG	ortho-Nitrophenyl-β-galactoside
oriV393	Short form of origin of replication from RK2-plasmid, containing 393 bp of *oriV* and the Rep-Sequence (P_{trfA}, *ssb*, *trfA)*
oriV632	Original form of origin of replication from RK2-plasmid containing the full 632 bp of *oriV* and the Rep-Sequence (P_{trfA}, *ssb*, *trfA)*
par	par sequence taken from the RK2 plasmid, containing *parA, parB parC, parD* and *parE*, Terminator $T_{13.2}$ at end of *parE* added via PCR
par29.1	par sequence taken from derived from the plasmid pCm470 Dsbc APLE C8P par29, containing *parA, parB parC, parD* and *parE* (last 50 bp are missing)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
REP	Origin of replication from pBBR1 (*Bordatella bronchiseptica*)
RFU	Relative fluorescing units
rpm	Revolutions per minute
RSF1010	Origin of replication from pRSF1010 (*Salmonella enterica)*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
TBS	Tris buffered saline
TSB	Tryptic soy broth medium

1 Introduction

1.1 *Ralstonia eutropha* H16

The gram-negative and respiratory facultative lithoautotrophic soil and freshwater bacterium *Ralstonia eutropha* H16 belongs to the family *Burkholderiaceae* of the β-Proteobacteria [1], [2]. In the past years *R. eutropha* H16, also known as *Alcaligenes eutrophus* and *Cupriavidus nector*, has attracted significant interest due to its ability to grow on H_2 and O_2 as sole energy source and to fixate CO_2 via the Calvin–Benson–Bassham cycle [3]. During absence of oxygen the organism can switch to denitrification by using nitrate as alternative electron acceptor [4]. Furthermore *R. eutropha* H16 is capable of producing and storing large amounts of poly[R-(–)-3-hydroxybutyrate] (up to 80% of its dry cell mass) in specialized storage granules during carbon excess and growth-limiting conditions like the lack of oxygen or nitrogen [5]. Therefore it became a favored organism for the industrial production of biodegradable polyesters.

For a better understanding of the remarkable versatility and its biotechnological potential the multiple replicon genome of *R. eutropha* H16 was analyzed [6], [7]. The complete nucleotide sequence consisting of three replicons - chromosome 1 (4,052,032 bp), chromosome 2 (2,912,490 bp), and the megaplasmid pHG1 (452,156 bp) - is known. Analysis of the distribution of genes from major functional categories revealed that most key functions for DNA replication, transcription and translation are encoded on chromosome 1, as well as important genes for the heterotrophic carbon metabolism. Typical substrates for heterotrophic growth are sugar acids like gluconic acid and other organic, fatty or amino acids.[6] Regarding the sugar metabolism necessary genes for the Entner-Doudoroff (KDPG) pathway are located on chromosome 2. However, the possible substrate spectrum of sugars for the H16 strain is limited to fructose [8]. The incapability of degrading glucose was justified with the lack of an active transport system [9].

Several genes for anaerobic growth on nitrate and nitrite as well as for the degradation of aromatic compounds had been located on chromosome 2 as well as on the megaplasmid pHG1 [7], [10]. In addition, the pHG1 megaplasmid harbors also important genetic determinants for two energy-conserving [NiFe]-hydrogenases of the H_2-oxidizing system: a cyto-

plasmic, NAD^+-reducing soluble hydrogenase (SH), and a dimeric membrane-bound hydrogenase (MBH) linked to the respiratory chain [11–13]. During lithotrophic growth *R. eutropha* H16 is also able to fixate CO_2 via the Calvin-Benson-Bassham (CBB) cycle. The genetic information of the key enzyme, the ribulose 1,5-bisphosphate carboxylase/oxygenase are encoded on duplicate *cbb* operons, located on the megaplasmid and on chromosome 2 [11], [14].

An effective regulatory system enables *R. eutropha* H16 to live under different habitant conditions and to switch between heterotrophic and autotrophic growth modes. This makes it a model organism for studying control mechanisms during autotrophic CO_2 fixation, hydrogen oxidation and denitrification. Furthermore, due to its ability to grow to high cell densities (up to 200 g dry weight/L) during litoautotrophic or heterotrophic conditions *R. eutropha* H16 has become a favored production organism with high biotechnological potential [15]. In order to use *R. eutropha* H16 for the expression of heterologous genes an appropriate regulatory expression system is required. Several attempts applying constitutive expression systems, based on broad-host-range plasmids and using the Tac or Lac promoter, for the expression of heterologous genes in *R. eutropha* H16 were already performed [16] [17] [18]. Due to the lack of transport capacities for inducers like lactose or IPTG expression systems were not inducible [8], [9]. However, other expression system employing different promoters like the P_{phaC} or P_{phaP} derived from *R. eutropha* H16 itself based on nitric oxide or hydrogen activation or the ace promoter induced by acetamidase were already reported [18–21]. Although these expression systems allow an inducible and tightly controlled expression the complex inducible systems prohibit their application as simple expression system in *R. eutropha* H16 under various growth conditions. In order to generate a simple expression system for *R. eutropha* H16 vectors using the P_{Tac} or the P_{Lac} as constitutive promoter were designed. The focus during this work was laid on the utilization of different origins of replication.

1.2 Origins of replication for expression vectors for *R. eutropha* H16

The basis for a successful and simple expression system for *R. eutropha* H16 are independently replicating elements – so called mini-replicons – which ensures the efficient repli-

cation and also the stably maintenance of the whole expression system in the desired strain. In order to be stably maintained and to minimize the metabolic load the replication of a plasmid has to be tightly controlled. Therefore the copy number of a plasmid is dependent on the given host and also on cell growth conditions. However, every particular plasmid has a characteristic copy number, which is achieved by plasmid-encoded control elements regulating the initiation of the replication [22]. The essential regions for a successful and tightly controlled replication harbors the following genes and loci: (i) the characteristic origin of replication (*ori*), (ii) the replication initiation protein (Rep protein) binding to cognate sites in the *ori* (which is not encoded in all plasmids), (iii) the plasmid-born genes involved in the control of the replication. In general, three different mechanisms for the replication of circular plasmid are known: the theta type, strand displacement and rolling circle. In gram-negative bacteria theta-replicating plasmids and strand displacement plasmids seemed to occur more often, whereas rolling circle plasmids are mainly derived from gram-positive bacteria [22].

The origins of replication used during this work originating from the RK2 (IncP family) and the RSF1010 (IncQ family) broad-host-range plasmids belong to the theta-type and the strand displacement type, respectively. The origin of replication from the pBBR1 broad-host-range plasmid does not belong to any of the incompatibility groups. It was also applied for the expression vector in *R. eutropha* H16. The replication mechanism of the pBBR1 plasmid is still unknown. In the next points the mechanisms of the applied origins of replications and their original plasmids are discussed in more detail.

1.2.1 REP origin of replication from pBBR1

The first origin of replication for the expression vectors for *R. eutropha* H16 originated from the broad-host-range plasmid pBBR1, which was isolated from the gram-negative bacterium, *Bordatella bronchiseptica.* The rather small plasmid (2.6 kb) consists of two functional cassettes: the region involved in plasmid replication, called Rep and the Mob region which is involved in mobilization processes. Sequence similarities between the Mob sequence and Mob/Pre proteins from plasmids of gram-positive bacteria led to the assumption that the replication mechanism of the Rep region may also be similar to the mechanism of gram-positive bacteria. However, no single-stranded intermediate form and no sequence similari-

ties between the pBBR1 sequences and sequences involved in the rolling-circle mechanism could be observed. Therefore the rolling-circle mechanism could be excluded from the possible replication mechanisms for the pBBR1 plasmid. Regarding the whole pBBR1 sequence a high overall G+C content was shown, with regions with exceptionally high A+T content in between. One of these A+T rich regions located upstream of the Rep open reading frame contained also two different pairs of direct repeat [23], [24]. This area seems to be involved in the replication, but further studies for the definitive identification of the replication mechanism of the pBBR1 plasmid are necessary.

Due to its medium copy number (up to 10 copies per cell in *Bordatella* species) the plasmid seems to be stably maintained in several gram-negative bacteria [23], [25], [26]. Via the addition of the pBluescript® II KS-LacZα-polylinker and several antibiotic resistance markers Kovach et al. constructed the pBBR1MSC derivates [16], [24]. They have been applied in a variety of gram-negative bacteria, like *R. eutropha* H16, for the development of genetic systems, generation of polyhydroxyalkanoates and biocatalysts [18], [27–29].

1.2.2 OriV393/632 from the RK2 broad-host-range plasmid

As second origin of replication for the set of expression vectors the oriV sequence from the broad-host-range RK2 plasmid was applied. The 60 kb RK2 broad-host-range plasmid (also known as RP1, RP4, R18 and R68) belongs to the IncPα incompatibility group [30], [31]. Due to its broad-host character the RK2 plasmid replicates and is stably maintained in a great variety of gram-negative bacteria. In *Escherichia coli* it replicates with a copy number of four to eight and in *Pseudomonas* of 2 to 3 [32]. Via the analysis of the complete nucleotide sequence the different coding regions, operons and genes from the RK2 plasmid were identified: the vegetative replication region (*oriV*) as well as a gene encoding replication initiation protein (*trfA*), regions for the conjugational transfer (*oriT, Tra*), a region encoding a multimer resolution system and postsegregational killing function (*psk/*mrs), a central control region (*ccr*) for encoding and combining partitioning functions and genes encoding for the three antibiotic resistance genes kanamycin, ampicillin and tetracycline [30], [33], [34]. All listed regions including specific genes and sequences are marked in *Figure 1.*

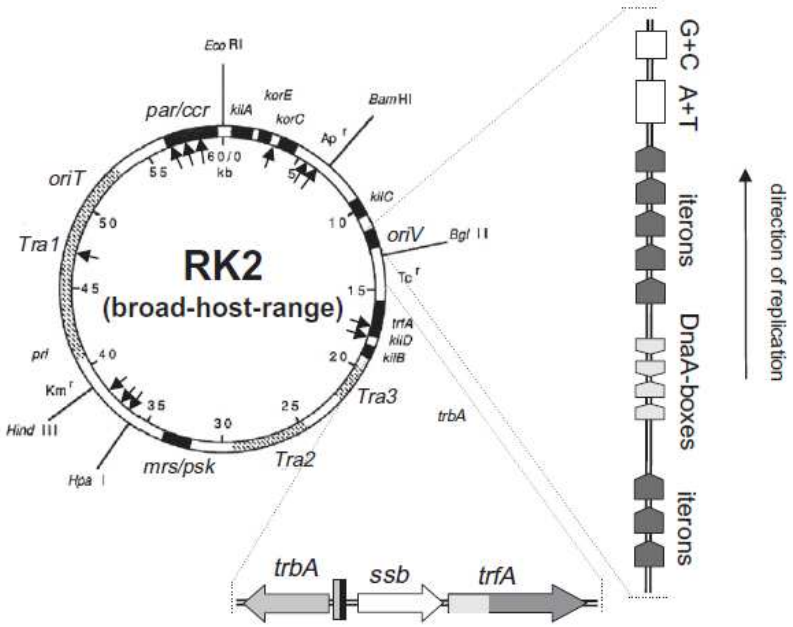


Figure 1: Map of RK2 plasmid; indicated are regions for plasmid replication, partitioning and stable maintenance: the vegetative replication region (oriV) with the gene for replication initiation protein (trfA), regions for the conjugational transfer (oriT, Tra), a region encoding a multimer resolution system and postsegregational killing function (mrs/psk), a central control region (par/ccr) for encoding and combining partitioning functions and three antibiotic resistance genes Km^r, Ap^r, Tc^r; On the right site: the replication motifs of the oriV consisting of four DnaA boxes for binding of DnaA protein during replication, eight repeated iteron sequences (sets of three and five) for binding the initiation protein trfA, the AT-rich region for the initial helix stabilization and helicase binding and the GC-rich region; At the bottom: the genes encoding the replication initiation protein (trfA) and a single strand binding protein (ssb) as well as the gene for global regulatory protein trbA; Picture taken from [35].

For the successful replication of the RK2 plasmid the region of the vegetative origin (*oriV*) the *trfA* (trans-acting replication function) locus upstream of the *oriV* encoding the replication imitation protein TrfA is required [34]. The whole regulation of the replication, partitioning and transfer system in the RK2 plasmid is tightly regulated via local control circuits and the global network controlled by the four repressors KorA, KorB, KorC (located in the *ccr*) and TrbA. [33] Additionally, a multimer resolution system is included via the Par/Mrs region which has efficient stabilizing effects on the RK2 plasmid during cell division in many gram-negative bacteria [30]. The genes of the *par* region will be discussed in more detail in the following chapter 1.3. A series of *kil* genes – either host-lethal (*kilA, B, C*) or interfering with plasmid maintenance (*kilD*) – and the corresponding *kor* genes for their regulation are located on the RK2 plasmid. Furthermore KorA/D and KorB seem to act as negative regulatory element for the *trfA* operon, controlling the copy number [36], [37]. However, neither Kil nor Kol seem to be essential for a successful plasmid replication. Studies revealed that a RK2-

minireplicon consisting only of *oriV* and the trfA operon *(trfA, ssb)* is able to replicate in many bacterial species, like *Escherichia coli* and *Pseudomonas putida* [35] [38].

After the determination of the nucleotide sequence of the minimal replicon a 617 bp *HaeII* restriction fragment was identified as oriV of the RK2 plasmid [39]. The replication mechanism of the RK2 plasmid belongs to the group of the theta-replicating plasmids. Within the oriV sequence eight 17-bp direct repeats (iterons) organized in two clusters of five and three iterons respectively are present. These highly conserved direct repeats are the binding sites for the replication initiation protein TrfA, providing a structural scaffold for the formation of replication initial complex [40]. Additionally, a 9 bp sequence serving as binding sites for the bacterial chromosome replication initiation protein DnaA is located between the two iteron clusters. For the initiation of DNA synthesis – opening of the double helix through initial destabilization - a specific region with a high adenosine and thymine content (AT-rich region) and low internal thermodynamic stability is required. The AT-rich region is located downstream from the 5-iteron, before the GC-rich region. Beside the oriV the *trfA* operon containing the trfA promoter P_{trfA}, the *trfA* gene and the coding sequence for a single strand binding protein ssb are necessary for replication and stable maintenance [39]. (See *Figure 1* and *Figure 2*) The trfA protein exists in two forms, differing in their molecular weight: 33 and 44 kDa. Both forms bind specifically to the two iteron clusters in the oriV and are active in plasmid origin opening [41–43]. In *E. coli* and *Pseudomonas putida* the smaller TrfA-33 was sufficient for plasmid replication, whereas *Pseudomonas aeruginosa* required the larger TrfA-44 protein for efficient replication [44].

Within the 617 bp oriV sequence a smaller 393 bp long HpaII restriction fragment was detected which is required for replication in *E. coli.* The 393-bp minimal origin consists only of the three essential regions for the initiation of replication: a cluster of five interons for binding the trfA protein, four DnaA binding boxes for binding the DnaA proteins and the AT-rich region serving as initial site for helix stabilization. The deletion of the set of three iterons located upstream of the minimal origin seemed to result in an increase of the copy number of the RK2 derivates [39], [45]. In Figure 2 the minimal replicon of the RK2 plasmid as well as the minimal origin functional in *E. coli* and *Pseudomonoas aeruginosa* are shown [35].

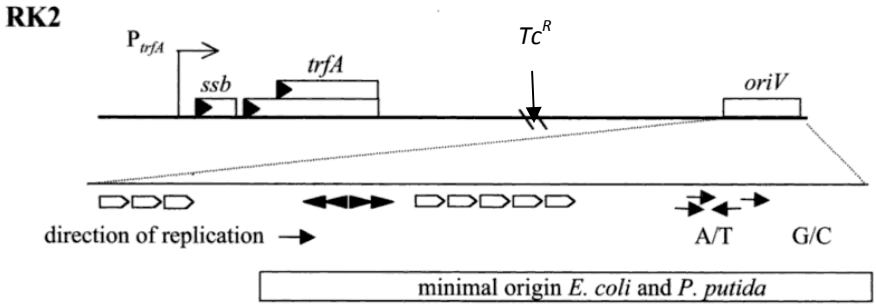


Figure 2: Minimal replicon of RK2 plasmid consisting of the oriV, the tetracycline resistance gene (marked with the black arrow) and the trfA operon. oriV: four DnaA boxes (black arrow box) for binding of DnaA protein during replication, eight repeated iteron sequences (sets of three and five, white arrow boxes) for binding the initiation protein trfA, the AT-rich region (black arrows) for the initial helix stabilization and helicase binding and the GC-rich region; trfA operon: promotor for trfA protein (P_{trafA}), genes of replication initiation protein (trfA) and of single-strand binding protein (ssb); For the minimal origin in E.coli and P. putida the cluster of three iterons located upstream in the oriV are not necessary; Picture taken from [35].

The 617 bp long *oriV* sequence of RK2 was already determined to be replicating in *R. eutropha* H16 [46]. Although the function of the smaller 393 bp oriV fragment was proved in *E. coli* and *P. putida*, the presence of the adjacent iteron cluster seemed to confer a stronger expression, tighter regulation of the copy number and an increased stability [45], [47], [48].

During this work both sequences of the oriV from the RK2 plasmid – the 617 bp and 393 bp sequence – were applied together with the trfA operon as origin of replication for expression vectors in *R. eutropha* H16.

1.2.3 RSF1010

The origin of replication the RSF1010 ori originating from the RSF1010 plasmid was applied for the expression vectors for *R. eutropha* H16. The small RSF1010 plasmid (8.7 kbp, similar to the R1161 plasmid) belongs to the IncQ incompatibility group. With a copy number of 10 to 12 copies per cell in *E. coli* the RSF1010 plasmid is a rather high copy number broad-host-range plasmid [49], [50]. The RSF1010 plasmid as well as its mini-replicon were already proved to replicate and stably maintained in *R. eutropha* H16 [13], [51], [52].

The basic structure of the RSF1010 replicon consists of two *trans*-acting regions and one *cis*-acting region, which are separated by mobilization genes and a transfer origin *oriT* [53]. The *cis* region containing the origin regulates replication and determines incompatibility. It consists of three and a half identical iterons of 20 bp, which serve as binding sites for the RepC protein, plus a 174-bp region with a GC-rich stretch of 20 bp and an AT-rich sequence of

31 bp. The structure of this region is similar to regions in the origin of the RK2 plasmid [22], [54], [55]. Additionally a large inverted repeat is present in the *cis* region, containing two plasmid-specific single- strand DNA initiation signals (marked as ssiA and ssiB) which are recognized by the RepB primase [56]. Three necessary proteins RepA, RepB, RepC for the replication initiation and for plasmid copy-number control are encoded in the two *trans* regions of the RSF1010 basic replicon [57]. The *repA* and *repC* are located in the same operon in the first *trans* region, whereas the nucleotide sequence for RepB and the larger form RepB* is harbored on another regulator unit in the second *trans* region. Rep A encodes for a helicase and possesses ATP dependent ssDNA binding activity, RepB have RSF1010 specific primase activity and RepC encodes for a dsDNA binding protein which regulates replication initiation by binding to the three direct repeats in the oriV [49], [58]. After the expression of the Rep proteins they cooperate with each other and bind to the *cis* region, containing the single-strand DNA initiation signals and the oriV. Between the *rep* genes the origin of conjugational DNA transfer *oriT* as well as the *mob* genes (ABC) encoding *trans*-active proteins for plasmid mobilization are located [59]. Furthermore the genes for two small proteins, designated as protein E and protein F, a control gene for the repA/C promoter also known as cac (= control gene for *repA* and *repC*) are located between *repA* and *repB*. The latter represents a repressor protein for the expression of *repA* and *repC,* hence regulating the initiation frequency of the oriV and the copy number of the RSF1010 plasmid. The function of protein E is still unknown [58], [60]. In *Figure 3* the map of the RSF1010 replicon with all mentioned genes and special regions is shown [35].

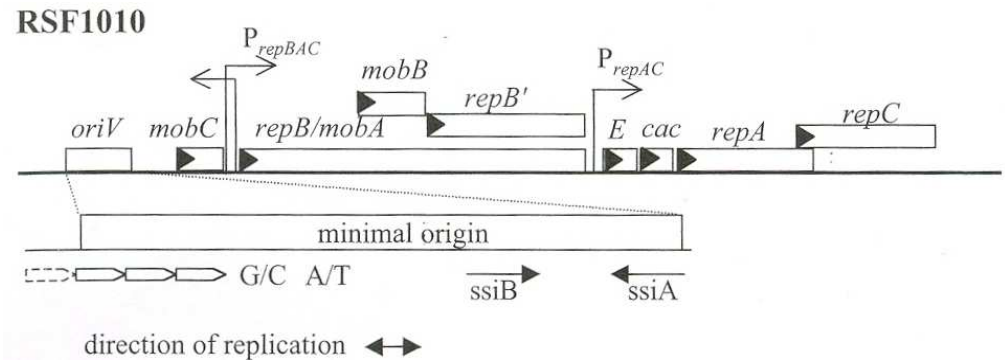


Figure 3: Map of the RSF1010 origin of replication: the origin of replication oriV sequence, containing the 20 bp iterons (white arrow boxes) for binding the RepC, the two single-strand DNA initiation signals (ssiA and ssiB) which are important for replication initiation, and the G/C and A/T rich region; the coding region for the replication initiation proteins RepA (helicase activity), RepB (primase activity), RepC (recognition of oriV), the origin of conjugational DNA transfer oriT, the mob genes mobA, mobB, mobC and the genes for the small proteins E (unknown function) and cac (repressor protein); picture taken from [35].

The RSF1010 replicon is replicated by the strand displacement mechanism. As initial step during the replication process the RepC protein binds to the iterons located in the origin and introduce conformational changes leading to DNA unwinding in the adjacent AT-rich region. During this DNA melting the plasmid-specific helicase RepA enters and unwinds the DNA of the flanking region, which contain the large inverted repeat with the two single-stranded DNA initiation site *ssiA* and *ssiB* [57]. The exposure of the *ssiA* and *ssiB* sites is necessary for the RepB primase to initiate replication. For the elongation of the replication the helicase activity of RepA is required. The combination of the three plasmid replication proteins RepA, RepB and RepC makes the replication initiation of the RSF1010 is independent of host replication factors like DnaA, DnaB, DnaC and DnaG [22].

Beside the necessary genes for replication the RSF1010 replicon carries also important genes encoding proteins for conjugative mobilization. The mobilization proteins MobA, MobB and MobC, as well as the transfer starting point, the origin of transfer (*oriT*), are encoded on the RSF1010 replicon [59], [61], [62]. During the mobilization process the DNA strand is knicked in the so-called *nic* site of the *oriT,* and the interrupted single DNA strand is than exported with its 5'end to a recipient cell [63] [64]. The MobA protein is responsible for the generation of the nic and forms a stable complex between the superhelical or linear RSF1010 DNA and the MobB and MobC proteins – the relaxation complex or relaxosome [65]. The binding of MobC, which is present in multiple copies on the relaxosome, cause a destabilization and opening of the double-stranded DNA helix. This facilitates the interaction of the MobA pro-

tein with the double stranded *nic* sequence and the cleavage of the active strand [65], [66]. The MobB protein seems to have a stimulartory function and its presence increases the yield of nicked protein [65].

However, plasmids carrying mobilization genes (Mob) and the *oriT* are not able to promote their own transfer unless an appropriate conjugation system is provided by a helper plasmid or is encoded on the chromosome of the donor strain. The additional proteins for the transfer (encoded via the *tra* genes) are involved in the formation of a pore or pilus, which enables the single-stranded DNA to enter the recipient strain [67]. A possible donor strain which has been already applied several times for the conjugation in *R. eutropha* H16 is the *Escherichia coli* S17-1 strain which has chromosomally integrated conjugal transfer functions, the *tra* genes from the RK2 plasmid [68–71] .

1.3 *Par*-Sequence from RK2 plasmid

In order to assure stable maintenance in the bacterial host strain plasmids utilize different control mechanisms, like copy number control, active partitioning systems and multimer resolution or postsegregational killing systems [72], [73]. Plasmids with lower copy numbers often inherit functions for an active partitioning system. First attempts applying the origin of replication of the pBBR1 plasmid in a new designed expression vector revealed some stability problems in *E. coli*. Furthermore, for the second origin of replication, the RK2 minireplicon, some maintenance problems have already been reported in literature [46], [74], [75]. Therefore an appropriate partitioning sequence facilitating the plasmid replication and improving the plasmid maintenance in *E. coli* and *R. eutropha* H16 was needed. The RK2 broad-host plasmid possesses such a partitioning sequence, the so called *par* region as already mentioned in *1.2.2*. Independent from its orientation the *par* region functions in several gram-negative bacteria. After cloning in different replicons, like the pBR322, its stabilizing effect resulted in 100% plasmid maintenance for more than 200 generation under nonselective growth conditions [74]. The *par* region consists of two operons *parABC* and *parDE* coding for five different proteins. The *parA* gene encodes a resolvase which can be translated from two different start codons, resulting in ParA1 or ParA2. For ParB endonuclease activity was determined, whereas the function of ParC is still unknown. Together these proteins

contribute to an active partitioning complex, which resolves plasmid multimers [76]. The *parDE* operon encodes a postsegregational killing system, which prevents the generation of plasmid-free segregants during cell division. The ParD protein serves as antitoxin for the neutralization of the toxic effects of the ParE protein [77]. In *Figure 4* the direction of the different *par* genes is shown.

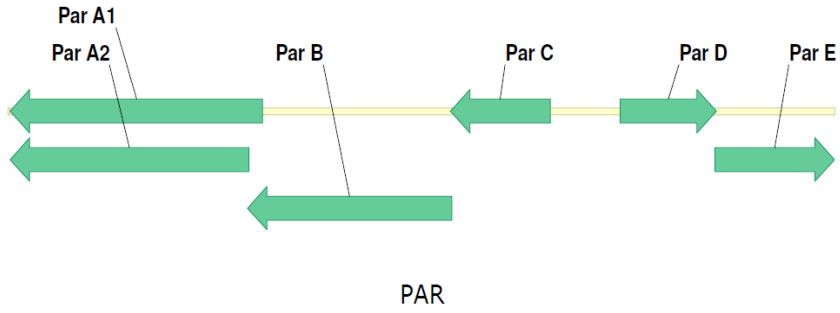


Figure 4: Schematic map of the par region from the RK2 plasmid: the region is organized in the two operons parABC and parDE encoding five proteins: ParA (encodes a resolvase), ParB (possesses nuclease activity), ParC (unknown function), ParD (antitioxin for ParE), ParE (toxic protein). The combination of ParA, ParB and ParC function as plasmid partitioning system, whereas ParD and ParE form a postsegregational killing system to ensures that plasmid-free daughter cells do not survive after cell division.

1.4 eGFP as reporter gene

The enhanced green fluorescence protein (eGFP) was selected as reporter gene in order to monitor the expression level of the designed expression vectors in *R. eutropha* H16. Several studies applying eGFP as reporter gene or using eGFP for the generation and detection of fusion proteins have been already accomplished [78–80].

EGFP is a modified variant from the wild type green fluorescent protein (GFP) which was isolated from the jellyfish *Aequorea victoria* [81]. The amino acid sequence of eGFP was modified in four positions compared to the wild type GFP in order to improve the intensity of the emission signal. The whole sequence was prolonged from 238 amino acids in the wild type GFP to 239 amino acids by adding a valine at position two. At position 64 and 65 a phenylalanine and a serine are exchanged with a leucine and threonine, respectively. At position 231 the last amino acid change in the sequence was performed via the replacement of a histidine with a leucine [82], [83]. Fluorescence microscopy using the optimum excitation wavelength of 488 nm is a quite fast and convenient method to proof the expression of the eGFP as reporter gene.

1.5 Aim of this work

The aim of this work was the construction of new expression vectors to facilitate heterologous gene expression in *R. eutropha* H16, with the main focus on the applied origin of replication. The research group of Petra Köfinger started with the construction of the pKR-Tac/Lac vectors, containing the REP origin of replication form the pBBR1 plasmid, the Tac or Lac promoter, the kanamycin resistance gene, the *lacI* repressor gene and *egfp* as reporter gene. However, due to the fact that *R. eutropha* H16 is lacking necessary transporter systems for sugars like lactose or the synthetic inducer IPTG an inducible expression system based on the Lac operon was not possible [8], [84]. Therefore the *lacI* gene was no longer required and removed from the vector. Furthermore, occurring stability and maintenance problems of the vectors in *E. coli* Top 10, as well as in *R. eutropha* H16 showed the necessity of a stabilizing partitioning system. The partitioning system from the RK2 broad-host plasmid, the *par*-region, was already proved to work very efficient in several gram-negative bacteria [74]. The *par* sequence was amplified from the pGMA29 plasmid, however, due to some unknown reasons the last 50 bp of the *parE* gene were missing in this plasmid [85].

As first part of this work the whole *par* sequence of the RK2 plasmid was directly amplified and integrated in the vector receiving the pKR-Tac-Par-eGFPΔlacI REP. Furthermore different origins of replication, varying in their copy number were integrated in the vector with the new *par* region. From the RK2 plasmid two forms of the minimal replicon – the oriV393 and the oriV632 – were applied as origins. The longer oriV632 consist of the whole vegetative origin (*oriV*) and the *trfA* operon and was already proved to replicate in *R. eutropha* H16, whereas the oriV393 lacks the sequence of a three iteron cluster [46]. As last origin of replication the mini-replicon of the RSF1010 rather high copy broad-host-range plasmid was employed. After the design of the expression vectors with the mentioned origins the plasmid replication and stably maintenance in *E. coli* Top10 and *R. eutropha* H16 was determined.

In the second part of this work the new designed vectors were tested for their ability to express the reporter protein eGFP. The amount of expressed protein was monitored via various detection methods, including SDS-PAGE, Western Blot analysis and the measurement of the fluorescing units.

2 Materials and Methods

2.1 Strains and plasmids

Table 1: Bacterial strains used in this work

Strain	Genotype	Source
***E. coli* TOP10**	F´(proAB, lacIq, LacZΔM15, Tn10(tet-r)), mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80ΔLacZΔM15, ΔlacX74, deoR, recA1, araD139(ara, leu), 7697, galU, galK, λ-, rpsL(streptomycin-r), endA1, nupG	Invitrogen
***R. eutropha* H16**	wildtype	DSM428
***E. coli* S17-1**	TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7	#679 IMBT strain collection

Table 2: Plasmids used in this work

Plasmid	Description	Source
RK2, RP4	Bla (amp^r), Kan^r, Tet^r, *par*, *oriV*, *oriT, mob, kil*	#381 IMBT strain collection
pJET1.2/blunt cloning vector	Bla (amp^r), P_{lacUV5}, Rep (pMB1), *eco47IR*, T7 promotor Cloning selection vector	Thermo scientific
pKR-Tac-*par29.1* -eGFP	Kan^r, P_{Tac}, REP, *egfp, par29.1*, *lacI* cloning vector for *E. coli* and *R. eutropha* H16	#6439 IMBT strain collection
pKR-Tac-*par29.1*-eGFPΔlacI	Kan^r, P_{Tac}, REP, *egfp*, *par29.1*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	#6440 IMBT strain collection
pRS415	Amp^r, *lac*Z, promoterless screening vector for *E. coli*	#6437 IMBT strain collection
pKT231	Kan-R, Sm-R, Mob+, RSF1010	#320 IMBT strain collection
pKR-Tac-Par-eGFPΔlacI REP	Kan^r, P_{Tac}, REP*, egfp*, *par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6799 IMBT strain collection)
pKR-Tac-Par-eGFPΔlacI oriV393	Kan^r, P_{Tac}, oriV393, *egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6798 IMBT strain collection)
pKR-Tac-Par-eGFPΔlacI oriV632	Kan^r, P_{Tac}, oriV632, *egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6797 IMBT strain collection)
pKR-Tac-Par-eGFPΔlacI RSF1010	Kan^r, P_{Tac}, RSF1010, *egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6814 IMBT strain collection)

pKR-Lac-Par-eGFPΔlacI Rep	Kan^r, P_{Lac}, Rep, *egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6813 IMBT strain collection)
pKR-ClV1a-Par-eGFPΔlacI Rep	Kan^r, P_{ClV1a}, REP, *egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6812 IMBT strain collection)
pKR-ClV1a-Par-ΔeGFPΔlacI Rep	Kan^r, P_{ClV1a}, REP, Δ*egfp, par*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6811 IMBT strain collection)
pKR-Tac-Par-LacZ(XbaI) ΔlacI Rep	Kan^r, P_{Tac}, REP, *LacZ* (cloned via *XbaI* and *HindIII*), p*ar*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6810 IMBT strain collection)
pKR-Tac-Par-LacZ(NdeI) ΔlacI Rep	Kan^r, P_{Tac}, REP, *LacZ* (cloned via *NdeI* and *HindIII*), p*ar*, *ΔlacI* cloning and expression vector for *E. coli* and *R. eutropha* H16	This work (#6809 IMBT strain collection)

2.2 Used primers

Table 3: Primers used in this work

Primer number refers to the internal primer list of the research group of Petra Köfinger. Bold characters mark restriction sites of endonucleases, whereas underlined characters mark the binding sites of the primer to the particular template.

Name	primer number	Sequence	TM [°C]
Rk2_Rep_overlap_fw	**190**	5‘-acgatgcttagctacgatccgctaaagttcttgacagcggaa-3‘	64,8
Rk2_Rep_term_*Pst*I_rev	**191**	5’-**ctgcag**aggacgaaaacgaaaagaggcagggccaagcgccccgcctcgaacagatatctagcgtttgcaatgcacc-3’	73,8
Rk2_oriV_*Spe*I _393 fw	**192**	5’– gg**actagt**ggcgacgtggagctgg-3’	67,7
Rk2_oriV_*Spe*I_632_fw	**193**	5’-gg**actagt**cctttccgacgctcacc-3’	65,1
Rk2_oriV_overlap_rev	**194**	5’-ggatcgtagctaagcatcgtccgggagggttcgagaa-3’	66,8
Rk2-ParA_*Spe*I_fw	**195**	5‘-gg**actagt**gcgaaaaggtgagaaaagccg-3‘	69,1
Rk2-ParE_rev	**196**	5’-tcagcccttgagcctgtcg-3’	69,4
RK2-ParE_Term_*Pst*I_rev	**197**	5‘-gg**actagt**gggcagggcatgaaaaagcccgtagcgggctgctacgggcgtctgacgcggtcagcccttgagcctgtcg-3‘	69,4
Par1_delta NdeI_rev	**137**	5'-acattagcacatgtgtgggcg-3'	68,8
Par2_delta NdeI_fw	**138**	5'-acgcccacacatgtgctaatg-3'	68,8

eGFP-fwd	**204**	5'-atggtgagcaagggcga-3'	66,2
eGFP-rev	**205**	5'-ttacttgtacagctcgtccatgc-3'	65,5
RK2-ParA_fw_*Pst*I	**233**	5'-aaa**ctgcag**gcgaaaaggtgagaaaagccg-3'	69,1
RK2-ParE_rev_*Not*I	**234**	5-ata**gcggccgc**gggcagggcatgaaaaagc-3'	69,1
LacZ_delta NdeI_fwd	**245**	5'-aatccccacatggaaaccg-3'	67,1
LacZ_delta NdeI_rev	**246**	5'-cggtttccatgtggggatt-3'	67,1
RSF1010_*Pst*I_rev	**263**	5'-**ctgcag**gagcagaagag-3'	57,9
RSF1010_*Spe*I_fw	**264**	5'-g**actagt**tcttcaaattcccgttg-3'	57,6
Rep_Seq4571_rev	**269**	5'-gtgggcggcctggaacgcgc-3'	81,6
Rk2-Rep_*Pst*I-short_rev	**270**	5'-**ctgcag**aggacgaaaacgaaaa-3'	54,5
eGFP_fwd_new	**294**	5'-taaacggccacaagttcagcg-3'	69,4
eGFP_rev_new	**295**	5'-aactccagcaggaccatgtgatc-3'	68,8
LacZ_*Nde*I_fwd	**328**	5'-cc**catatg**accatgattacgg-3'	64,0
LacZ_delta Nde_rev NEU	**329**	5'aatccccatgtggaaaccg-3'	67,1
LacZ_delta Nde_fwd NEU	**330**	5'-cggtttccacatggggatt-3'	67,1
LacZ_*Hind*III_rev	**331**	5'-cc**aagctt**tttttgacaccag-3'	63,8
SD+insert pKR-tac/lac-par	**332**	5'-ccggaattctttaagaaggag-3'	61,0

2.3 General protocols

2.3.1 Growth media and conditions

The cultivation of *E. coli* Top10 strains was performed at 37°C using regular LB Media (Carl Roth GmbH CO. KG; Heidelberg, Germany) with 100 µg/mL ampicillin or 40 µg/mL kanamycin, depending on the resistance gene of the particular plasmid. *R. eutropha* H16 strains were cultivated at 28°C, employing different media with 200 µg/mL kanamycin and 20 µg/mL gentamycin. Tryptic soy broth (TSB, BD Diagnostic Systems; Heidelberg, Germany) media was used for the cultivation on agar plates and for fermentations. Minimal salt media (MSM) [86], regular LB media and nutrient broth media (NB) were used for fermentations in liquid media. The composition of the different media is listed in *Table 4*.

Table 4: Composition of different growth media

Media	Composition
LB	Yeast extract (5 g/L), Trypton (10 g/L), Sodium chloride (0,5–10 g/L)#
TSB	Bacto Trypton (17.0 g/L), Bacto Soyton (3.0 g/L), Dextrose (2.5 g/L), Sodium chloride (5.0 g/L), Di-potassium hydrogen phosphate (2.5 g/L); pH 7.3 ± 0.2
MSM	Solution A (30x): Na_2HPO_4 x12 H_2O (270 g/L), KH_2PO_4 (45 g/L)
	Solution B (30x): $MgSO_4$ x $7H_2O$ (6 g/L), NH_4Cl (60g /L), Fe(III)NH_4-Citrat (125 mg/L), SL7 (30 mL)
	Solution C (100x): $CaCl_2$ x $2H_2O$ (250 mg), ad 250 mL
	Trace elements solution (SL7): 25 %(w/v) HCl (1.3 mL/L), H_3BO_3 (62 mg/L), $CoCl_2$ x $6H_2O$ (190 mg/L), $CuCl_2$ x $2H_2O$ (17 mg/L), $MnCl_2$ x $4H_2O$ (100 mg/L), Na_2MoO_4 x $2H_2O$ (36 mg/L), $NiCl_2$ x $6H_2O$ (24 mg/L), Zn Cl_2 (70 mg/L); pH 6,5
	Mix solution A and B with bidest. H_2O (1:1:28), pH7.0; after autoclaving add 0.01 volume of solution C
NB	Peptone (5 g/L), meat extract (3 g/L)

All standard chemicals are from Carl Roth GmbH CO. KG. Exceptions are mentioned in the text.

2.3.2 Preparation of competent cells of *E. coli* Top10 and *R. eutropha* H16

For the preparation of competent *E.coli* Top10 cells 15 mL LB media were inoculated with a *E. coli* Top10 single colony and incubated over night at 37°C at 110 rpm. The main culture (500 mL LB media, 2 L flask) was inoculated to an OD_{600} of 0.1 and incubated at 37°C at 110 rpm till an OD_{600} of 0.7-0.8. After reaching the right OD_{600} the cells were cooled on ice for 30 min and then centrifuged at 4°C for 15 min at 4500 rpm (centrifuge Avanti^{TM} J-20 XP, JA-10 Rotor, Beckmann Coulter, Inc.; Vienna, Austria). The supernatant was removed, the pellet was resuspended in 5 mL ice-cold water and 250 mL water was added. Afterwards the cells were centrifuged for 15 min at 2500 rpm. This step was repeated twice. After the last centrifugation step the supernatant was removed and the pellet was resuspended in 5 mL of ice-cold glycerol (10 %) and another 20 mL glycerol were added. After centrifugation for 15 min at 4500 rpm the supernatant was removed 2-3 mL glycerol were added to resuspend the pellet. Portions of 90 µL were filled into 1.5 mL Eppendorf tubes and frozen in liquid nitrogen. The competent cells were stored at -80°C.

For the preparation of competent *R. eutropha* H16 cells the preculture (30 mL TSB media with 20 µg/mL gentamycin, 50 mL flask with a triangular magnetic stirrer) was inoculated with a glycerol stock from *R. eutropha* H16 and incubated at 28°C at 500 rpm overnight. The

main culture (100 mL TSB media, 300 mL flask with a triangular magnetic stirrer) was inoculated to an OD_{600} of 0.1 and incubated at 28°C at 500 rpm till an OD_{600} of 0.8-1.0. After reaching the right OD_{600} the cells were cooled on ice for 30 min and then centrifuged in a 50 mL flacon at 4°C for 15 min at 4000 rpm (centrifuge 5810 R, Eppendorf; Hamburg, Germany). The supernatant was removed and the pellet was resuspended in 5 mL of 0.3 M ice-cold sucrose. After centrifuging again at 4°C for 10 min at 4000 rpm the pellet was washed in 2.5 mL of 0.3 M sucrose. After the last centrifugation for 5 min at 4°C at 4000 rpm the cells were diluted with ~1 mL of 0.3 M sucrose to reach an OD_{600} about 30. The OD_{600} was measured with the Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).Portions of 100 µL were filled into 1.5 mL Eppendorf tubes and stored at -80°C.

2.3.3 Transformation in competent *E. coli* Top10 and *R. eutropha* H16 cells

For the transformation of DNA in *E.coli* Top10 cells 40 µL of electrocompetent cells were mixed with 100-300 ng DNA and incubated on ice for 10 min. Transformation was performed using the program EC2 (0.2 mm cuvettes, 2.5 kV) of Micro Pulser™ (Bio-Rad; Hercules, USA). Afterwards 1 mL of LB media was added to the cells which were then incubated at 37°C at 750 rpm in a thermomixer for 45-60 min. After regeneration 100 µL were plated out and the rest of the cells was centrifuged for 1 min at maximum speed and resuspended in 100 µL LB media. For plating out LB-plates with the appropriate antibiotic (Kanamycin 40 µg/mL or Ampicillin 100 µg/mL) were applied.

For the transformation in *R. eutropha* H16 cells an aliquot of 100 µL competent cells was mixed with 100-300 ng DNA and incubated on ice for 30 min. Transformation was performed with the EC2 program of Micro Pulser™. After the electroporation the cells were regenerated in 1 mL of TSB media and incubated for 2 h at 28°C and 900 rpm. Afterwards the cells were centrifuged for 5 min at 4000 rpm and resuspended in 100 µL TSB media and plated out on TSB plates containing 200 µg/mL kanamycin and 20 µg/mL gentamycin.

2.3.4 Conjugation in *R. eutropha* H16

The conjugation in *R. eutropha* H16 using the *E.coli* S17-1 as donor strain was only performed for the expression vector containing the RSF1010 origin of replication. This replicon possesses the *mob* genes for conjugative mobilization in addition to the replicative func-

tions. The *tra* genes for the formation of a pilus between donor and recipient strain are integrated in the chromosome of the *E.coli* S17-1 donor strain and enable the conjugative plasmid transfer. For the cultivation of the donor strain *E. coli* S17-1 [pKR-Tac-Par-eGFP-ΔlacI RSF1010] 10 mL of LB medium with 40 µg/mL kanamycin were inoculated with a single colony and incubated for about 15 h at 30°C and 110 rpm. The recipient strain *R. eutropha* H16 was cultivated in 10 mL of TSB medium with 20 µg/mL gentamycin and incubated over night at 30°C and 110 rpm. After the cultivation the donor and recipient cells were harvested via centrifugation in 50 mL grainer tubes for 15 min at 4000 rpm at 4°C. Afterwards the cell pellets were resuspended in 500 µL of 0.9 % NaCl solution. For the conjugative plasmid transfer 0.2 mL of the donor suspension as well as 0.2 mL of the recipient suspension were spotted on the middle of an TSB agar plate. The plates were incubated for about 20 h at 30°C. Additional control plates with either 0.2 mL donor suspension or 0.2 mL recipient suspension were also incubated. For the selection of the positive transconjugates – *R. eutropha* H16 containing the pKR-Tac-Par-eGFP-ΔlacI RSF1010 vector – the grown cells from the conjugation plate were transferred to TSB agar plates containing 20 µg/mL gentamycin and 200 µg/mL kanamycin. The donor cells are not able to grow on gentamycin, whereas empty recipient cells would not survive the high level of kanamycin. The cells grown on the conjugation plate were resuspended in 3 mL of 0.9 % NaCl solution and then diluted stepwise to 10^{-5}. From each dilution step 0.1 mL were plated out on the TSB plates with 20 µg/mL gentamycin and 200 µg/mL kanamycin. From these plates single colonies were picked and used for a single streak out.

2.3.5 General cloning protocols

Restriction digest and dephosphorylation:

All used restriction enzymes and corresponding buffers were ordered from Thermo Scientific (Waltham, Massachusetts, USA).

Preparation of restriction mixture:

x µL	200-500 ng DNA
1.5 µL	reaction buffer (10x)
0.5 -1 µL	restriction enzyme (10 U/µL)
y µL	bidest. H_2O to a final reaction of 15 µL
15 µL	

The restriction mixture was incubated at 37°C for at least 3 h. For preparative restrictions higher amounts of DNA (~500 ng) and 1 µL of enzyme (10 U/µL) were applied, using a higher end volume (20-25 µL). The mixture was incubated overnight at 37°C. The reaction was stopped via thermal inactivation of the enzyme. The particular temperatures are given by Thermo Scientific.

Dephosphorylation with Shrimp Alkaline Phosphatase (SAP):

x µL	0.5 – 1 µg of linear DNA
2 µL	SAP buffer (10x)
1 µL	SAP (1 U/µL)
y µL	bidest. H_2O to a final reaction of 20 µL
20 µL	

The mixture was incubated at 37°C for 15 min and then 1 µL of SAP was added additionally and incubated for another 15 min. The reaction was stopped via the thermal inactivation of the SAP at 65°C for 15 min.

PCR:

For general PCR reaction the Phusion™ High-Fidelity DNA Polymerase from Finnzymes (Vantaa, Finnland) was applied. The PCR set-up was based on the protocol provided by the company. The basic components for the PCR reaction are listed in *Table 5.*

Table 5: Basic reaction components for PCR with a total volume of 25 and 50 µL

Component	Total volume of 25 µL	Total volume of 50µL
H_2O	Add to 25 µL	Add to 50 µL
5x Phusion HF buffer	5 µL	10 µL
10 mM dNTPs	0.5 µL	1 µL
Primer A	1.25 µL	2.5 µL
Primer B	1.25 µL	2.5 µL
Template DNA	10-15 ng	10-15 ng
Phusion DNA polymerase (2 U/µL)	0.3 µL	0.5 µL

For the PCR program (see *Figure 5*) the initial denaturation and final extension the conditions are provided by the manufacturer. As thermocycler the GeneAmp® PCR System 2700 from Applied Biosciences (Norwalk, Connecticut, USA) was used.

Initial denaturation	98°C	30 sec	
Denaturation	98°C	30 sec	25 cycles
Annealing	X°C	20 sec	
Extension	72°C	Y sec	
Final extension	72°C	10 min	

Figure 5: General PCR program

The annealing temperature depends on the primer length and on the GC content. For its calculation the TM finnzymes calculator was employed. Regarding the extension time the amplicon length and complexity has to be considered. For low complexity DNA like plasmids 15 sec per 1 kb were used.

In *Table 6* the primer pairs with the particular annealing temperature and extension time for all standard PCRs performed during this work are listed.

Table 6: PCR conditions for all standard PCRs

Name of PCR product	Template	Primer pairs	Annealing temperature	Extension time	Number of cycles
ParA-NdeI	RK2 plasmid	Rk2-ParA_*Spe*I_fw Par1_delta NdeI_rev	68 °C	30 sec	25
NdeI-ParE	RK2 plasmid	Par2_delta NdeI_fw Rk2-ParE_rev	68 °C	15 sec	25
oriV 393 bp sequence	RK2 plasmid	Rk2_oriV_*Spe*I _393 fw Rk2_oriV_overlap_rev	66 °C	10 sec	25
oriV 632 bp sequence	RK2 plasmid	Rk2_oriV_*Spe*I _632 fw Rk2_oriV_overlap_rev	66 °C	10 sec	25
Rep-sequence from RK2	RK2 plasmid	Rk2_Rep_overlap_fw Rk2_Rep_term_*Pst*I_rev	65 °C	25 sec	25
RSF1010	pKT231	RSF1010_*Spe*I_fw RSF1010_*Pst*I_rev	57 °C	120 sec	25
***lac*Z (Δ*Nde*I) fragment 1**	pRS415	LacZ_*Nde*I_fwd LacZ_delta Nde_rev NEU	67 °C	50 sec	25
***lac*Z (Δ*Nde*I) fragement 2**	pRS415	LacZ_delta Nde_fwd NEU LacZ_*Hind*III_rev	67 °C	15 sec	35
***lac*Z (Δ*Nde*I) with E*coR*I+*Hind*III**	pKR-Tac-Par-LacZ-ΔlacI Rep	SD+insert pKR-tac/lac-par LacZ_*Hind*III_rev	67°C	50 sec	25
***lac*Z (Δ*Nde*I)**	pKR-Tac-Par-LacZ-ΔlacI Rep	LacZ_*Nde*I_fwd LacZ_*Hind*III_rev	67°C	50 sec	25

Overlap extension PCR:

An overlap extension PCR is a special PCR method for the introduction of specific base exchanges in a sequence or for the splicing of two DNA fragments.

In order to insert a specific base exchange four primers had to be designed, the two outer primers, which were binding to the beginning and the end of the sequence and two inner primers. These inner primers have to bind to the region were the base exchange should be introduced and contained the desired new base. In the first step two standard PCRs had to be performed. For the generation of the first PCR product the sequence from the beginning until the position of the desired mutation was amplified with the outer forward and the inner reverse primer. In the second PCR the sequence from the point of the mutation to the end of the sequence was amplified applying the inner forward and the outer reverse primer. During this step the inner primers generated a complementary overlap containing already the desired mutation at the 3' end of the first PCR product and at the 5' end of the second

PCR product. In the second step the products (molar ratio 1:1, total DNA concentration ~70 ng) of the standard PCRs served as templates for the overlap extension PCR. In the first six cycles the PCR reaction was performed without primers for the sticking together of the two templates with their complementary ends, containing the desired base exchange and to fill up the complementary strands. Then the outer primers were added for another 18 cycles for the amplification of the whole sequence.

The principal for the splicing of two different DNA fragments is quite similar. First two standard PCRs had to be performed, amplifying the desired fragments and generating a complementary overlap at the 3' end of the first fragment and at the 5' end of the second fragment. Therefore an overlap sequence of 20 bp was added to the reverse primer of the first and to the forward primer of the second fragment. After performing the standard PCRs the two products served as templates for the overlap extension PCR. In the first six cycles the PCR reaction was performed without primers for the sticking together of the two templates with their complementary ends. Then the outer primers were added for another 18 cycles for the amplification of the whole sequence.

All PCR reactions were performed using the Phusion™ High-Fidelity DNA Polymerase from Finnzymes. The basic components for the PCR reaction mix are listed in *Table 5* and the temperature program is similar to the program shown in *Figure 5*, except for the cycle number. For the overlap extension PCR the first 6 cycles were conducted without primers and another 18 cycles were run after adding the outer primers. The PCR conditions for the standard PCRs are shown in *Table 6* and the conditions for the overlap extension PCRs in *Table 7*.

Table 7: PCR conditions for all overlap extension PCRs

Name of PCR product	Template (1. and 2.)	Primer pairs	Annealing temperature	Extension time	Number of cycles
oriV393	oriV 393 bp sequence and Rep-sequence from RK2	Rk2_oriV_*Spe*I _393 fw Rk2_Rep_term_*Pst*I_rev	64 °C	30 sec	6 x (no primer) 18x
oriV632	oriV 632 bp sequence and Rep-sequence from RK2	Rk2_oriV_*Spe*I _632 fw Rk2_Rep_term_*Pst*I_rev	64 °C	30 sec	6 x (no primer) 18x
par sequence	ParA-NdeI and NdeI-ParE	Rk2-ParA_SpeI_fw RK2-ParE_Term_*Spe*I_rev	68 °C	35 sec	6 x (no primer) 18x
***lac*Z (Δ*Nde*I) with *Nde*I+*Hind*III**	LacZ (ΔNdeI) fragment 1 LacZ (ΔNdeI) fragment 2	LacZ_NdeI _wd LacZ_HindIII_rev	67 °C	50 sec	6 x (no primer) 18x

Colony PCR for *egfp*:

As sample for a colony PCR eiteher a colony from an agar plate or 20-50 µL of an liquid culture were used. From the colony a small tooth pick tip was directly resuspended in 20 µL of bidest. H_2O whereas the liquid culture was first centrifuged and then the obtained pellet was resuspended in 20 µL of bidest. H_2O. After boiling the sample for 10 min at 99°C it was centrifuged for 1 min at max. speed. Then 2 µL of the supernatent were employed as template for the colony PCR. For the reaction the DreamTaq DNA Polymerase from Thermo Scientific was applied. The conditions for the colony PCR reaction for *egfp* for a total volume of 25 µL are listed in *Table 8.*

Table 8: Reaction components for colony PCR for egfp with a total volume of 25 µL

Component	Total volume of 25 µL
H_2O	Add to 25 µL
10x DreamTaq buffer	2.5 µL
10 mM dNTPs	0.5 µL
DMSO	0.75 µL
Primer #294	1.25 µL
Primer #295	1.25 µL
Template DNA	2 µL
Dreamtaq DNA polymerase (5 U/µl)	0.2 µL

For the colony PCR temperature program the initial denaturation and final extension the conditions are provided by the manufacturer. Regarding the annealing temperature and the extension time the conditions depend on the primer and template characteristics. As PCR machine the GeneAmp® PCR System 2700 from Applied Biosciences (Norwalk, Connecticut, USA) was used. In *Figure 6* the temperature program of the colony PCR is shown.

Initial denaturation	95°C	3 min	
Denaturation	95°C	30sec	25 cycles
Annealing	58°C	30 sec	
Extension	72°C	45 sec	
Final extension	72°C	10 min	

Figure 6: Temperature program of the colony PCR for egfp

Agarose gel:

For the preparation of a 1% agarose gel 2 g agarose (Biozyme; Vienna, Austria) were mixed with 200 mL of 1xTAE buffer (Stock solution 50x: 242 g/L Tris, 14.6 g/L EDTA, 57.1 g/L acetic acid) and heated in the microwave until the agarose was dissolved (~ 3 min). After cooling 2-3 drops of EtBr were added and the gel was poured into the gel tray. The samples were mixed with 6x Loading Dye+SDS (Thermo Scientific) and then loaded onto the gel. As standard 5 µL of the GeneRuler™ DNA ladder (for map see *Figure* 7) were applied.

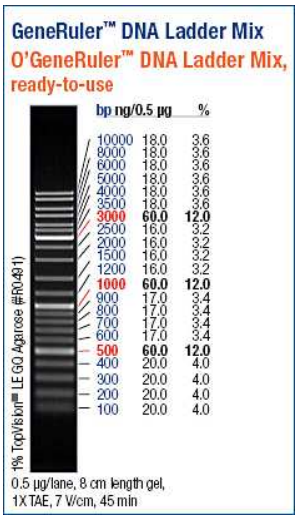


Figure 7: The GeneRuler™ DNA ladder Mix.

For a control gel the voltage was set 110-120 V and the gel was run for 45 min. In case of a preparative gel the voltage was lowered to 90 V and the running time was extended to 90-120 min. For detection the GelDoc-It™ Imaging System from UVP (Cambridge, UK) was used.

Wizard® SV Gel and PCR Clean-Up System Kit:

In order to clean up a PCR reaction product or a restriction digest to remove disturbing reaction compounds or enzymes and for the dissolving of an agarose gel slice the Wizard® SV Gel and PCR Clean-Up System Kit from Promega (Mannheim, Germany) was applied.

A. Dissolving of the gel slice

Add 10 µL of membrane binding solution per 10 mg of gel slice
Vortex and incubate sample at 65°C until the gel slice is completely dissolved.

B. Processing PCR reactions

1. Add an equal volume of membrane binding solution to the PCR reaction

C. Binding of DNA

1. Insert SV Minicolumn into the collection tube
2. Transfer the dissolved gel mixture or the prepared PCR product into the minicolumn assembly and incubate at room temperature for 1 min
3. Centrifuged for 1 minute at 16,000 x *g*. Discard the flowthrough and reinsert the minicolumn into the collection tube.

D. Washing
1. Add 700 µL of membrane wash solution, centrifuged for 1 min at 16,000 x *g*. Discard the flowthrough and reinsert the minicolumn into the collection tube.
2. Repeat step D.1 with 500 µl of membrane wash solution, centrifuged for 5 min at 16,000 x *g*. Discard the flow-through and reinsert the minicolumn into the collection tube.
3. Centrifuge again for 1 minute at 16,000 x *g*.

E. Elution
1. Transfer the minicolumn into a clean 1.5 ml microcentrifue tube.
2. Add **30 µl** of bidest. H_2O and incubated at room temperature for **5 min**.
Centrifuge for 1 min at 16,000 x *g*.

Changes in the manufacturer's protocol were marked with bold letters.

Ligation:

For the ligation the Promega T4 DNA Ligase (Promega; Mannheim, Germany) was used, following the manufacturer's protocol. Regarding the vector to insert ratio normally a ratio of 1:3 or 1:2 was applied.

Ligation mixture:

x µL	~ 70 ng vector DNA
y µL	appropriate amount of insert DNA
1.5 µL	T4 DNA ligase buffer (10x)
1 µL	T4 DNA ligase (1 U/µL)
z µL	bidest. H_2O to a final reaction of 15 µL
15µL	

The ligation mixture was incubated at 16°C overnight. Afterwards the ligase was inactivated at 65°C for 20 min. Before transformation a desalting of the ligation mixture is necessary in order to prevent any short-circuit. Therefore nitrocellulose filters (MF^{TM} Membrane Filters, 0.025 µm VSWP, Merck Millipore; Billerica, Massachusetts, USA) were put in a petri dish filled with water. The whole ligation mixture was pipetted on the filters, swimming on the water surface, and incubated for 20-30 min.

GeneJET Plasmid Miniprep Kit:

For the isolation of plasmids the GeneJET Plasmid Miniprep Kit from Thermo Scientific was applied:

1. Resuspension of cells, lysis and neutralization
Add to the pelleted cells:
* 250 µL of resuspension solution and vortexed
*250 µL of lysis solution and inverted tube 4 – 6 times
*350 µL of neutralization solution and inverted tube 4 – 6 times
Centrifuge mixture for **10 min** at full speed

2. Binding of DNA
Load supernatant to GeneJET™ spin column and centrifuge for 1 min at full speed

3. Washing of column
Add 500 µL of wash solution and centrifuge for 1 min at full speed } Repeat twice
Discharge the flowthrough
Centrifuge empty column for 1 min at full speed

4. Elution of purified DNA
Transfer column into a new tube.
Add **30-50 µL** of bidest.H_2O to column and incubate for **5 min**
Centrifuge for 2 min at full speed, collect flow through in fresh Eppi

Changes to the manufacturer's protocol were marked with bold letters.

Thermo Schientific CloneJET™ PCR cloning Kit:

For the cloning of PCR products into the pJET vector the Thermo Schientific CloneJET™ PCR cloning Kit from Thermo Scientific was used.

For the ligation the appropriate amount of PCR product (0.15 pmol of DNA ends) had to be determined using *Table 9.*

Table 9: Recommended amount of PCR product for the ligation reaction

Length of PCR product (bp)	Optimal PCR product quantity (0.15 pmol ends)
100	5 ng
300	15 ng
500	25 ng
1000	50 ng
2000	100 ng
3000	150 ng
4000	200 ng
5000	250 ng

Ligation mixture:

x µL	~ 0.15 pmol ends of purified PCR product/other blunt-end DNA fragment
10 µL	reaction buffer (2x)
1 µL	pJET1.2/blunt Cloning Vector (50 ng/µL)
1 µL	T4 DNA ligase (5 U/µL)
y µL	bidest. H_2O to a final reaction of 20 µL
20µL	

The ligation mixture was incubated at room temperature for 10-15 min, depending on the size of the particular PCR product. Afterwards the ligation mixture was directly used for transformation in *E.coli* Top10 cells.

Sequencing:

Sequencing was performed in order to confirm the correct sequence of the generated PCR products. Therefore plasmid DNA was isolated and 10 µL (~100 ng/µL) were sent together with 4 µL of the respective primer (5 µM) for sequencing to LGC Genomics GmbH (Berlin, Germany). In case of the pJET1.2 cloning vector system the primers were provided by LGC Genomics.

2.4 Construction of the expression vectors

2.4.1 Construction of pKR-Tac-Par-eGFPΔlacI REP

For the construction of the pKR-Tac-Par-eGFPΔlacI REP vector the already designed pKR-Tac-*par29.1*-eGFPΔlacI REP vector was used. This vector contained the Tac Promotor, the REP sequence from the pBBR1-MCS5 plasmid as origin of replication, the kanamycin resistance gene, *egfp* as reporter gene and the *par29.1* sequence which was taken from the pGMA29 plasmid [85]. (See *Figure 8*)

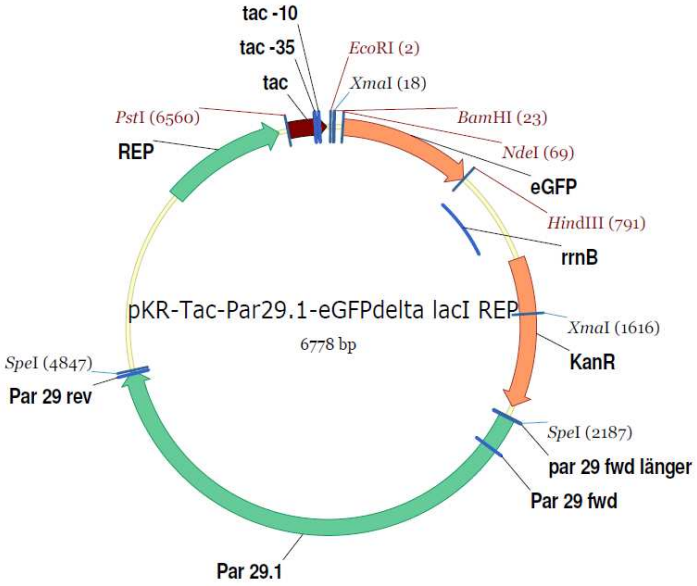


Figure 8: Vector map of pKR-Tac-par29.1-egfpΔlacI REP, containing the Tac promoter, the REP origin of replication, the kanamycin resistance gene, egfp as reporter gene and the old par29.1 sequence from the pGMA29 plasmid.

The *par29.1* acts as partitioning sequence which supports the plasmid distribution during cell division and prevents plasmid loss. The detailed sequence of the *par* region and the different functions of its genes were already mentioned in *1.3*. However, through blast search it was discovered that in the *par29.1* sequence from the pGMA the last 50 bp of the original *par*E gene as well as a terminator sequence are missing.

Therefore, the aim of this part was the construction of a new expression vector containing the whole sequence of the *par* region with all genes as well as a concluding terminator sequence on the end of *parE*. Both, the whole *par* region as well as the terminator ($T_{L13.2}$) (sequence is shown in *Appendix 3*: Sequences) were taken from the RK2 broad-host-range plasmid [30]. Additionally to the new terminator sequence also two *Spe*I restriction sites on both ends of the *par* sequence was added for the cloning of the new sequence in the old vector backbone. However, for the amplification of the *par* sequence from the RK2 plasmid an overlap extension PCR was applied in order to delete an *Nde*I restriction site between *par*C and *par*D. In both expression vectors the desired reporter gene was cloned via an *Nde*I and *Hind*III restriction site therefore all additional *Nde*I sites in the vector backbone had to be removed.

For the overlap extension PCR five different primers were designed. The two inner primers were binding in the region of the *Nde*I site and contained one different base pair in the *Nde*I

sequence. The inner forward primer (#138 Par2_fw) contained a cytosine instead of a tyrosine base and the inner reverse primer (#137 Par1_rev) a guanidine instead of an adenine. Through this base exchange a silent mutation was introduced thus the amino acid sequence was preserved. (See *Figure 9*)

5'...CA^TA TG...3´ → 5´…CACATG…3´

3'...G T A T^AC...5´ → 3´…GTGTAC…5´

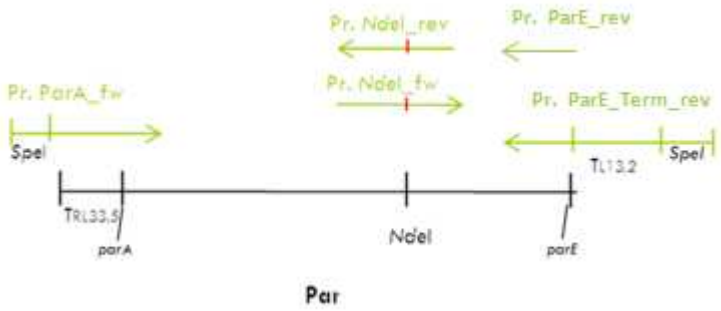
Figure 9: Base pair exchange for the deletion of the NdeI restriction site between parC and parD

The outer forward primer (#195 Rk2_ParA_*Spe*I_fw) was binding to the 5' end of the *par* sequence attaching a *Spe*I restriction site to the end of *par*A. As outer reverse primer two primers were designed, the first one for the standard PCR for the fragment from the deleted *Nde*I site to the end of *par*E (#196 Rk2_ParE_rev) and the second one for the overlap extension PCR (#197 RK2_ParE_Term_*Pst*I_rev) containing the $T_{L13.2}$ terminator sequence and a *Spe*I restriction site to the end of *par*E.

In order to amplify the first fragment ranging from *par*A to the deleted *Nde*I site and the second fragment from the deleted *Nde*I site to *par*E via standard PCR the primer pairs #195 and #137 respectively #196 and #138 were employed. For the amplification of the second fragment the short outer primer was used, in order to amplify just the sequence until the end of *par*E. For the standard PCR conditions see *Table 6*. Afterwards the two PCR mixtures were loaded onto an agarose gel and an the appropriate bands were cut out and cleaned up with the Wizard® SV Gel and PCR Clean-Up System Kit.

The two PCR products were then combined via the overlap extension PCR. The first six cycles were run without the primers, just for the sticking together of the two fragments. Afterwards the two outer primers (#195 Rk2_ParA_*Spe*I_fw and #197 RK2_ParE_Term_*Spe*I_rev) were added and another 18 cycles were conducted. Applying the longer outer reverse primer the $T_{L13.2}$ terminator sequence and the *Spe*I restriction site were now attached to the end of *par*E. For the PCR conditions see *Table 7*. In *Figure 10* (A) the primer binding sites and their characteristics are marked on a schematic *par* sequence, whereas *Figure 10* (B) shows the desired overlap extension product including the different *par* genes with the new attached terminator sequence and *Spe*I restriction sites.

(A)



(B)

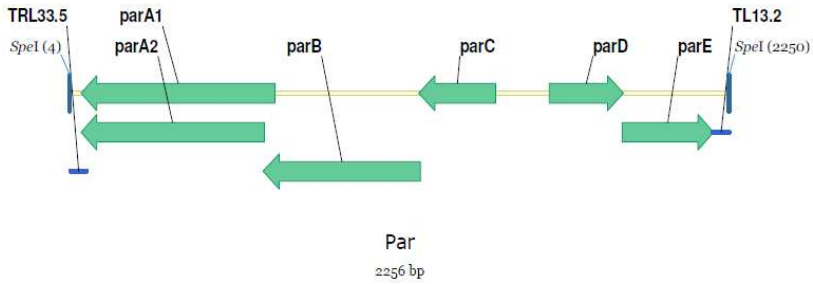


Figure 10: Scheme for the overlap extension PCR for the par sequence from the RK2 plasmid (A) and scheme of the par sequence marking the different genes and the new SpeI restriction sites (B). ParA encodes a resolvase, parB has nuclease activity and the function of parC is not known yet. ParD and parE form a killing system for plasmid free segregants, in which parE encodes a lethal polypeptide and parD an antagonist.

Afterwards the PCR product was loaded on an agarose gel. The appropriate band was cut out and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. The PCR product was cloned into the pJET vector system and positive clones were sent for sequencing.

After positive sequencing results the pJET vector containing the *par* sequence and the vector pKR-Tac-*par29.1*-eGFPΔlacI REP were cut with *Spe*I and the restriction mixtures were loaded on an agarose gel. The desired bands (vector backbone: 4121 bp, Par-sequence: 2246 bp) were cut out of the gel and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. Afterwards the vector backbone was ligated with the *par* sequence and transformed in *E.coli* Top10. The transformants were streaked out for plasmid isolation which was then cut with *Spe*I in order to control the presence of the new Par-sequence.

The resulting new vector was named pKR-Tac-Par-eGFPΔlacI REP (see *Figure 11)* and was transformed into *R. eutropha* H16.

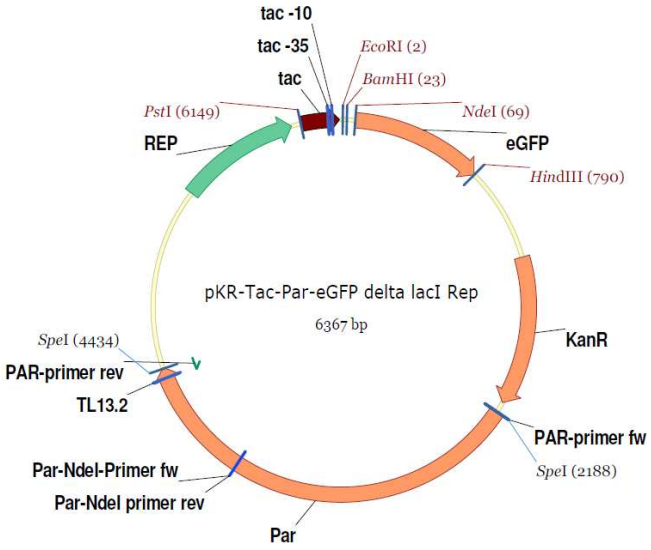


Figure 11: Vector map of pKR-Tac-Par-egfpΔlacI REP, containing the Tac promoter, the REP origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designed par sequence.

2.4.2 Construction of pKR-Tac-Par-eGFPΔlacI oriV393/oriV632

For the construction of the pKR-Tac-Par-eGFPΔlacI oriV393/632 vector the REP origin of replication had to be replaced with the oriV393 or oriV632 from the RK2 plasmid [30]. However, it was not possible to use the new designed pKR-Tac-Par-eGFPΔlacI REP vector because the exchange of the origins via cutting with *Spe*I and *Pst*I would also involve the removal of the Par-sequence, which was cloned into the vector via the *Spe*I restriction site.

Therefore, the pKR-Tac-*par29.1*-eGFP REP vector (see *Figure 13* (A)) containing the Tac-promotor, the REP origin of replication, the *par29.1* sequence, the kanamycin resistance gene, *egfp* as reporter gene and the *lac*I gene between the *Pst*I and *Not*I restriction was used. First the *par29.1* had to be removed via cutting with *Spe*I. After re-ligation and transformation of the pKR-Tac-Δ*par29.1*-eGFP REP vector (see *Figure 13* (B)) into *E.coli* Top10 the plasmid was isolated again and cut with *Pst*I and *Not*I to remove the *lac*I gene. Between these two restriction sites the new *par* region should be integrated. Therefore, the *par* sequence was amplified via PCR from the pKR-Tac-Par-eGFPΔlacI Rep with new designed primers (#233 RK2-ParA_fw_*Pst*I and #234 RK2-ParE_rev_*Not*I), which attached a *Pst*I and *Not*I restriction site to the end of *par*A and of *par*E (see *Figure 12*). The PCR program is shown in *Table 6.*

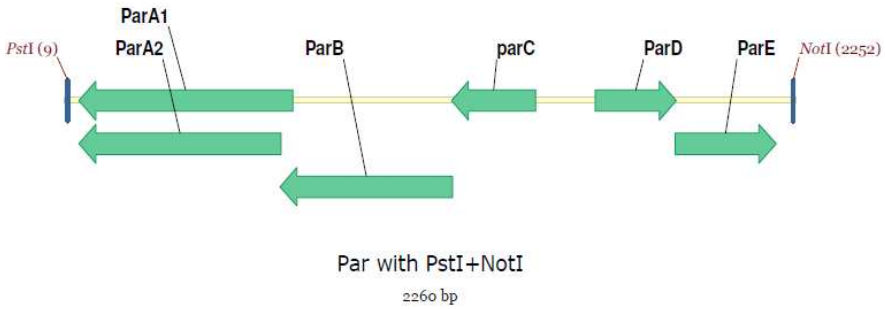


Figure 12: Par sequence from RK2 plasmid with PstI restriction site on the end of parA and NotI restriction site on the end of parE.

The PCR product as well as the pKR-Tac-Δ*par29.1*-eGFP REP vector were cut with *Pst*I and *Not*I and loaded on an agarose gel. The appropriate bands (vector backbone: 4118 bp, Par-sequence: 2260 bp) were cut out and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. After ligating vector backbone and *par* sequence the ligation mixture was transformed into *E. coli* Top10. Ten transformants were streaked out for plasmid isolation and the plasmids were isolated and cut with *Pst*I and *Not*I in order to confirm the presence of the Par-sequence. All steps for the construction of the pKR-Tac-Par-eGFPΔlacI REP with *par* between *Pst*I and *Not*I are illustrated in *Figure 13*.

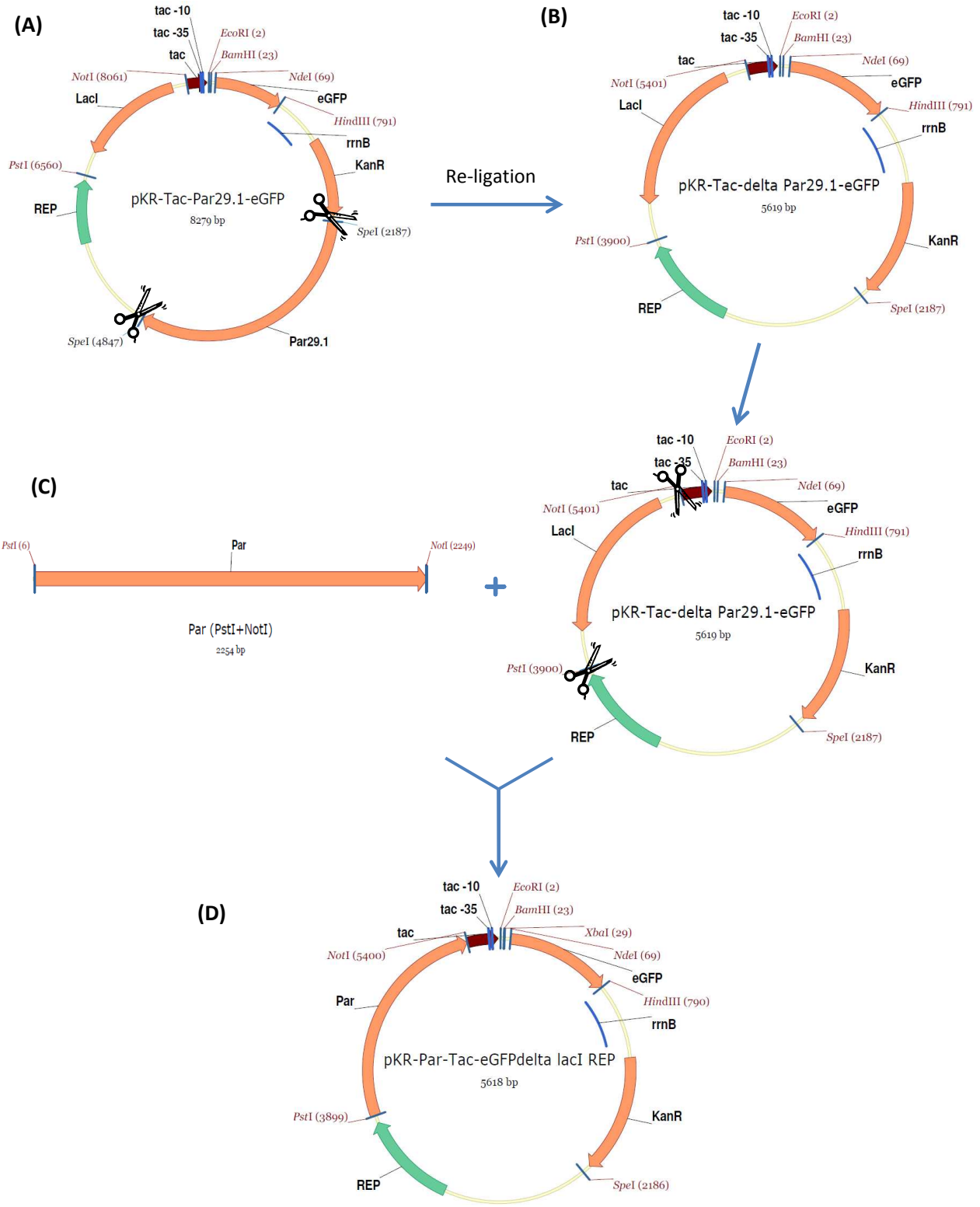


*Figure 13: Scheme for the construction of the pKR-Tac-Par-egfpΔlacI REP with the par sequence between the PstI and NotI restriction sites: The pKR-Tac-par29.1-egfp **(A)** was cut with SpeI to remove the par29.1 sequence and then re-ligated. The resulting pKR-Tac-Δpar29.1-egfp REP **(B)** was cut with PstI and NotI to remove the lacI and was then ligated with the new designed par sequence with PstI and NotI restriction sites **(C)**. The new constructed pKR-Tac-Par-egfpΔlacI REP **(D)** was then used for the construction of pKR-Tac-Par-egfpΔlacI oriV393/oriV632.*

Due to the fact that the *par* sequence in this new designed pKR-Tac-Par-eGFPΔlacI Rep vector was located between *Pst*I and *Not*I the exchange of the origin of replication could now be performed via cutting with *Spe*I and *Pst*I.

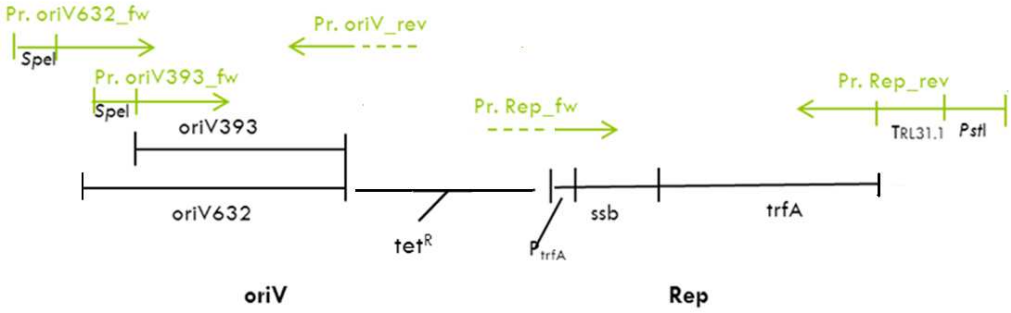
The new origins oriV393 and oriV632, originating from the RK2 plasmid were designed via overlap extension PCR. On the RK2 plasmid the *oriV* sequence and the Rep sequence gene (including the *trf*A binding protein gene, the ssb sequence and the *trf*A promotor) are separated via a tetracycline resistance gene. Regarding the *oriV* two different sequences varying in length (393 bp and 632 bp) are described in literature to work together with the Rep-sequence as minimal replicon [39], [87]. Therefore, two origins of replication out of the RK2 plasmid were amplified: the oriV393, containing 393 bp of the *oriV* sequence and the Rep sequence and the oriV632, containing the 632 bp of the *oriV* sequence and the Rep sequence. In order to remove the tetracycline resistance gene and to combine the sequences of the *oriV* and Rep two standard PCRs were performed attaching an complementary overlap to the 3' end of the particular *oriV* fragment and to the 5' end of the Rep-sequence.

For the first fragments (oriV with 393bp or 632 bp) the two forward primers (#192 Rk2_oriV_*Spe*I _393 fw or #193 Rk2_oriV_*Spe*I_632_fw) and the reverse primer (#194 Rk2_oriV_overlap_rev) were applied. The forward primers add a *Spe*I restriction site to the 5' end and the reverse primer the designed overlap to the 3' end of the *oriV* sequence. The second fragment, the Rep-sequence was amplified with a forward primer (#190 Rk2_Rep_overlap_fw) attaching the designed overlap to the 5' end and with a reverse primer (#191 Rk2_Rep_term_*Pst*I_rev) which add the terminator sequence $T_{RL31.1}$ (sequence is shown in *Appendix 3*: Sequences) from the RK2 plasmid and a *Pst*I restriction site to the 3' end [30]. For the standard PCR conditions see *Table 6*. Afterwards the two PCR mixtures were loaded onto an agarose gel and the appropriate bands were cut out and cleaned up with the Wizard® SV Gel and PCR Clean-Up System Kit.

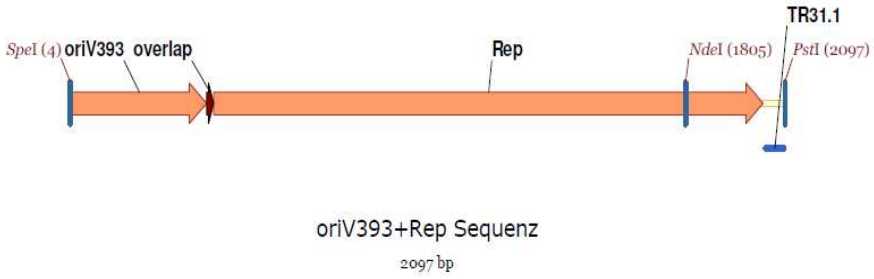
The fragments of the *oriV* (393 bp or 632 bp) were both combined with the amplified fragment of the Rep-sequence during the overlap extension PCR. The first six cycles were run without primers, just for sticking together the two fragments. Afterwards the particular outer primers (#192 or #193 and #191) were added and another 18 cycles were conducted. For

the overlap extension PCR conditions see *Table 7*. In *Figure 14* (A) the primer binding sites and their characteristics are marked on a schematic overview of the *oriV* and Rep-sequence. *Figure 14* (B) and (C) show the desired overlap extension products including the new attached terminator sequence $T_{RL31.1}$ and the *Pst*I restriction site on the 3' end of the Rep-sequence and the *Spe*I restriction site on the 5' end of the particular *oriV* sequence.

(A)



(B)



(C)

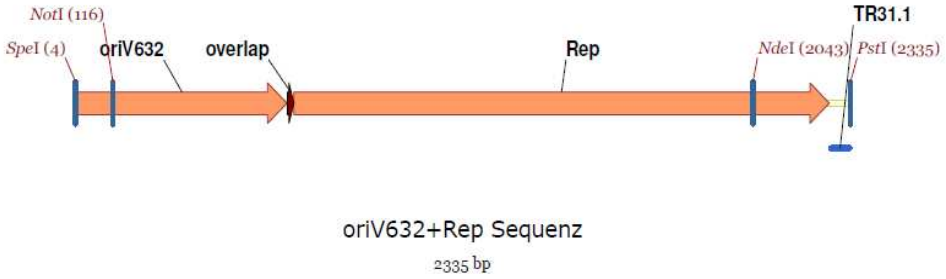


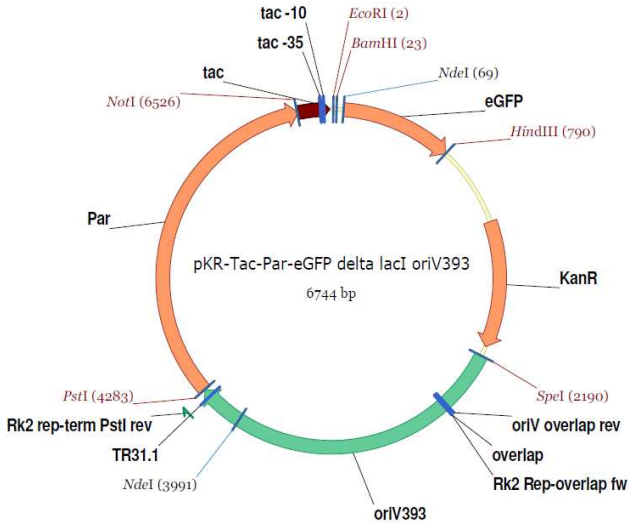
Figure 14: Scheme for the overlap extension PCR of the oriV sequence and the Rep sequence on the RK2 plasmid (A) and scheme of the new designed origins oriV393 (B) and oriV632 (C) with the added $T_{RL31.1}$ terminator sequence and the SpeI and PstI restriction sites.

Afterwards the PCR products were loaded onto an agarose gel. The appropriate bands were cut out and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. The PCR products were cloned into the pJET vector system and positive clones were sent for sequencing. After positive sequencing results the pJET vector containing the oriV393 or oriV632 and the designed pKR-Tac-Par-eGFPΔlacI REP vector with *par* between *Pst*I and *Not*I (see *Figure*

13 (D)) were cut with *Spe*I and *Pst*I. The restriction mixtures were loaded onto an agarose gel and the desired bands (backbone: 4.600 bp, oriV393: 2097 bp, oriV632: 2335 bp) were cut out of the gel and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. Then the vector backbone was ligated with the particular origin and transformed in *E.coli* Top10. After isolating the vector from the gained transformants it was cut again with *Spe*I and *Pst*I in order to control the presence of the oriV393 or oriV632.

The resulting new vectors pKR-Tac-Par-eGFPΔlacI oriV393 (see *Figure 15 (A))* and pKR-Tac-Par-eGFPΔlacI oriV632 (see *Figure 15* (B)) were transformed in *R. eutropha* H16.

(A)



(B)

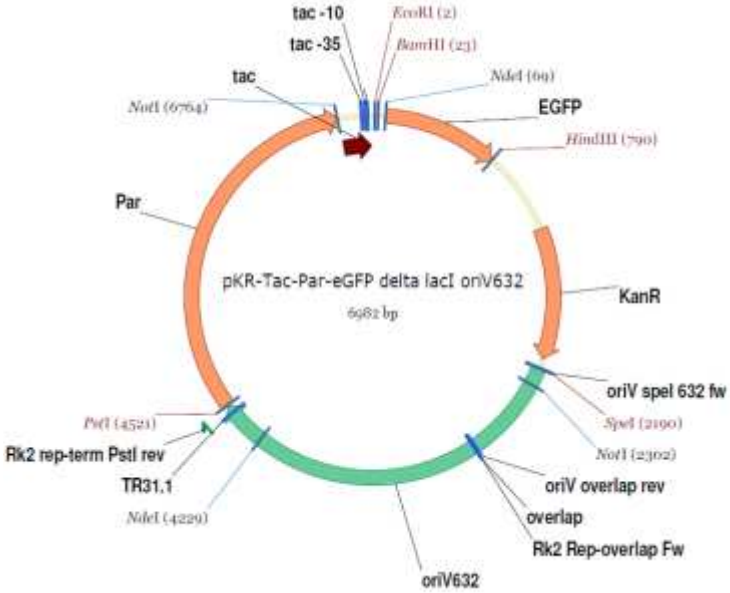


Figure 15: (A) Vector map of pKR-Tac-Par-egfpΔlacI oriV393, containing the Tac promoter, the oriV393 origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designed par sequence. (B) Vector map of pKR-Tac-Par-egfpΔlacI oriV632, containing the Tac promoter, the oriV632 origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designed par sequence

2.4.3 Construction of pKR-Tac-Par-eGFPΔlacI RSF1010

For the construction of the pKR-Tac-Par-eGFPΔlacI RSF1010 vector the pKR-Tac-Par-eGFPΔlacI oriV393 (see *Figure 15* (A)) containing the Tac promoter, the oriV393 origin of replication, the par sequence located between *Pst*I and *Not*I, the kanamycin resistance gene and *egfp* as reporter gene was used. The vector was cut with *Spe*I and *Pst*I in order to re-

move the old origin oriV393 and to insert the new origin RSF1010 from the pKT231 plasmid [51].

The RSF1010 was directly amplified from the pKT231 plasmid via standard PCR, attaching a *Pst*I restriction site via the forward primer (#263 RSF1010_*Pst*I_rev) at the 5' end and *a Spe*I restriction sites via the reverse primer (#264 RSF1010_*Spe*I_fw) on the 3' end of the sequence. The whole sequence of the RSF1010 and all its genes are already shown in *Figure 3*. For the PCR conditions see *Table 6.*

The PCR product of the RSF1010 and the pKR-Tac-Par-eGFPΔlacI oriV393 vector were cut with *Pst*I and *Spe*I and the restriction mixtures were loaded onto an agarose gel. The desired bands (vector backbone: 4600 bp, RSF1010: 5743 bp) were cut out of the gel and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. Then the vector backbone was ligated with the RSF1010 and transformed in *E.coli* Top10. After isolating the vector from the gained transformants it was cut again with *Spe*I and *Pst*I in order to control the presence of the RSF1010 and then transformed into *R. eutropha* H16.

The resulting new vector was named pKR-Tac-Par-eGFPΔlacI RSF1010 and is shown in *Figure* 16.

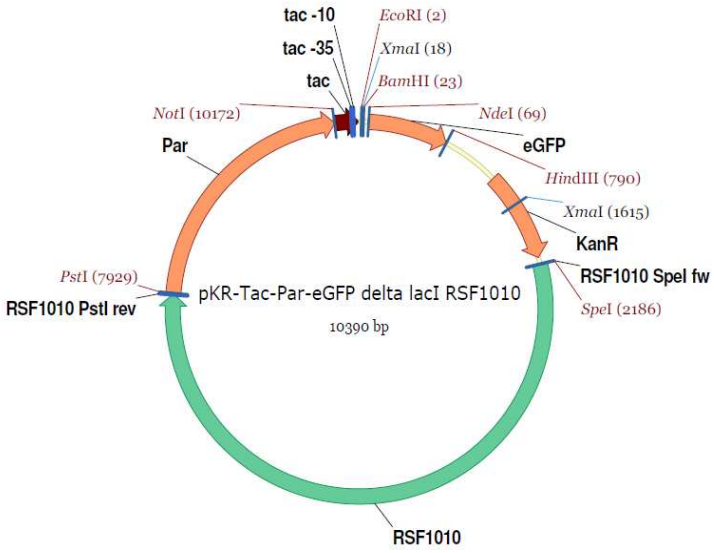


Figure 16: Vector map of pKR-Tac-Par-egfpΔlacI RSF1010, containing the Tac promoter, the RSF1010 as origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designed par sequence.

Due to the presence of the *mob* genes in the RSF1010 replicon the new vector was not only transformed into *R. eutropha* H16 via electro transformation, but also introduced into *R. eutropha* H16 via conjugation applying *E. coli* S17-1 as donor strain.

2.4.4 Construction of pKR-Tac-Par-lacZΔlacI REP

For the construction of the pKR-Tac-Par-LacZΔlacI REP vector the pKR-Tac-Par-eGFPΔlacI REP vector (see *Figure 11*), containing the Tac-promotor, the REP origin of replication, the *par* sequence located between two *Spe*I restriction sites, the kanamycin resistance gene and *egfp* as reporter gene was used. To create the new expression vector the *egfp* should be replaced with the *lacZ* from the pRS415 plasmid (for vector map see ***Fehler! Verweisquelle konnte nicht gefunden werden.***, using the *Xba*I and *Hind*III restriction sites.

The pRS415 plasmid and the pKR-Tac-Par-eGFPΔlacI REP were cut with *Xba*I and *Hind*III and the restriction mixtures were loaded onto an agarose gel. The desired bands (pKR-Tac-Par-ΔeGFPΔlacI REP vector backbone: 5606 bp and *lacZ*: 3078 bp) were cut out and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. Then the pKR-Tac-Par-ΔeGFPΔlacI REP vector backbone was ligated with the *lacZ* and transformed in *E.coli* Top10. After isolating the plasmid from the gained transformants they were cut again with *Xba*I and *Hind*III to control the proper insertion of *lacZ*.

The resulting new vector was named pKR-Tac-Par-LacZΔlacI REP (see *Figure 17*) and was transformed into *R. eutropha* H16 for further experiments.

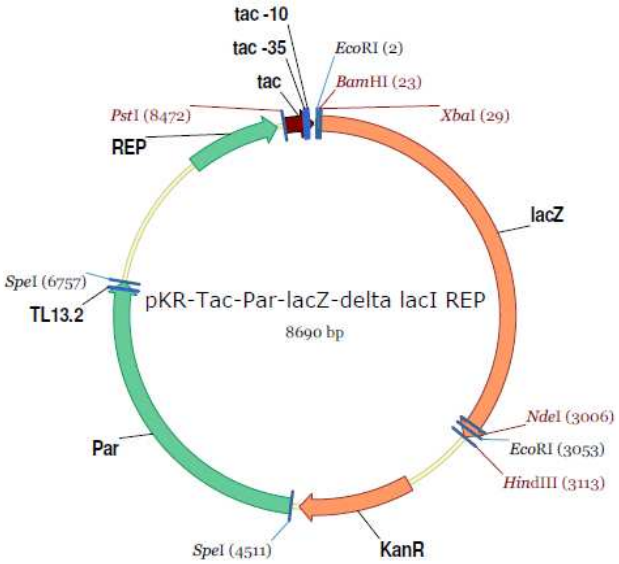


Figure 17: Vector map of pKR-Tac-Par-lacZΔlacI REP, containing the Tac promoter, the REP as origin of replication, the kanamycin resistance gene, lacZ as reporter gene (cloned with XbaI and HindIII) and the new designed par sequence.

However, by cutting the pKR-Tac-Par-eGFPΔlacI REP with *Xba*I the Shine Dalgarno sequence which is located just in front of the *Nde*I restriction site was removed. This may cause problems for the expression of *lacZ*. Therefore, the second approach was performed through cloning *lacZ* into the pKR-Tac-Par-eGFPΔlacI REP vector via the *Nde*I and *Hind*III restriction sites. However, the *lacZ* contained an *Nde*I restriction site, which had to be mutated via an overlap extension PCR.

The *lacZ* sequence was directly amplified from the pRS415 plasmid applying four different primers. The two inner primers were binding in the region of the *Nde*I site and contained one different base pair in the *Nde*I sequence. The inner forward primer (#330 LacZ_delta Nde_fwd NEU) contained a cytosine instead of a tyrosine base and the inner reverse primer (#329 LacZ_delta Nde_rev NEU) a guanidine instead of an adenine. Via this base exchange a silent mutation was introduced, thus the amino acid sequence was preserved. (See *Figure 18*)

5'...CA^TA TG...3´ → 5´…CACATG…3´

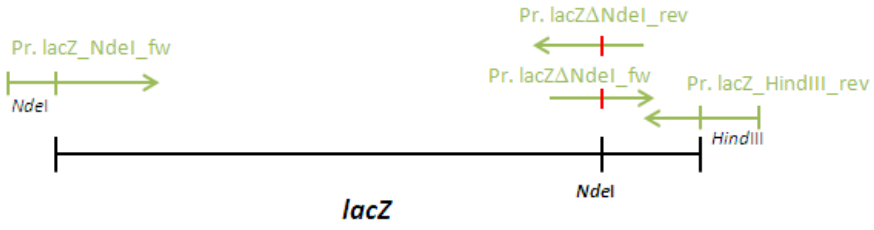
3'...G T A T^AC...5´ → 3´…GTGTAC…5´

Figure 18: Base pair exchange for the deletion of the NdeI restriction site in the lacZ sequence.

The outer forward primer (#328 LacZ_*Nde*I_fwd) was binding to the 5' end of *lacZ* attaching a *Nde*I restriction site, whereas the outer reverse primer (#331 LacZ_*Hind*III_fwd) was binding to the 3' end of *lacZ* attaching a *Hind*III restriction site. In order to amplify the first fragment from the 5' end of l*acZ* to the deleted *Nde*I site and the second fragment from the deleted *Nde*I site 3' end of *lacZ* via standard PCR the primer pairs #328 and #329 respectively #330 and #331 were employed. For the standard PCR conditions see *Table 6.* Afterwards the two PCR mixtures were loaded onto an agarose gel and the appropriate bands were cut out and cleaned up with the Wizard® SV Gel and PCR Clean-Up System Kit.

The two PCR products were combined via the overlap extension PCR. The first six cycles were run without primers, just for the sticking together of the two fragments. Afterwards the two outer primers (#328 and #331) were added and another 18 cycles were conducted. For the conditions of the overlap extension PCR see *Table 7*. In *Figure 19* (A) the primer binding sites and their characteristics are marked on a schematic *lacZ* sequence. The mutation for the *Nde*I restriction site in the sequence of the inner primers is marked in red. *Figure 19* (B) shows the sequence of the mutated *lacZ* with the *Nde*I and *Hind*III restriction sites at the 5' and 3' end.

(A)



(B)

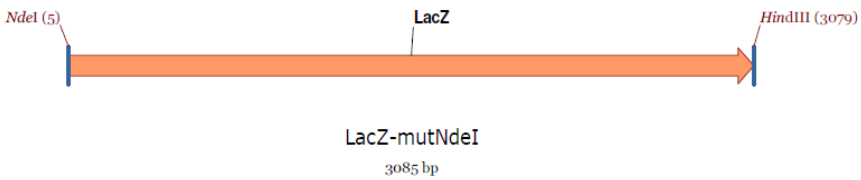


Figure 19: Scheme for the overlap extension PCR for the lacZ sequence from the pRS415 plasmid (A) and scheme of lacZ marking the new NdeI and HindII restriction sites (B).

Afterwards the PCR product was loaded on an agarose gel. The desired band was cut out and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. The cleaned PCR product was cloned into the pJET vector system and positive clones were sent for sequencing.

After positive sequencing results the pJET vector containing the *lacZ* and the vector pKR-Tac-Par-eGFPΔlacI REP were cut with *Nde*I and *Hind*III and the restriction mixtures were loaded onto an agarose gel. The appropriate bands (vector backbone: 5650 bp, *lacZ*: 3122 bp) were cut out of the gel and cleaned up using the Wizard® SV Gel and PCR Clean-Up System Kit. Then the vector backbone was ligated with the *lacZ* and transformed in *E.coli* Top10. 10 transformants were streaked out for plasmid isolation of the particular plasmids. These were cut with *Nde*I and *Hind*III to check the presence of *lacZ*. Afterwards the new designed vector was transformed into *R. eutropha* H16.

The resulting new vector was named pKR-Tac-Par-LacZΔlacI Rep and is shown in *Figure 20*.

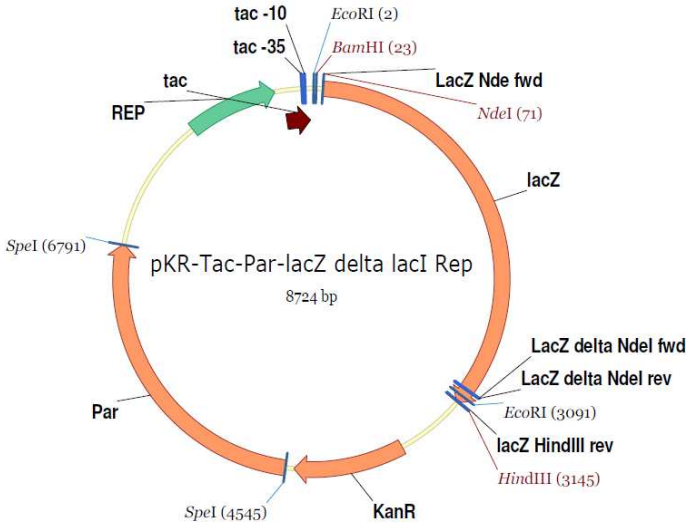


Figure 20: Vector map of pKR-Tac-Par-lacZΔlacI REP, containing the Tac promoter, the REP origin of replication, the kanamycin resistance gene, the par sequence and lacZ as reporter gene.

2.5 Plasmid stability assay

In the new designed pKR-Tac vectors the *par* region from the broad host range RK2 plasmid was introduced to enhance the distribution of the plasmids during cell division and to prevent plasmid loss. All pKR-Tac-Par-eGFPΔlacI vectors containing the different origins of replication were transformed into *E. coli* Top10 and *R. eutropha* H16 cells and the stability of the plasmids in both strains was proven by performing the following plasmid stability assay.

For the *E. coli* Top10 strains a liquid overnight culture inoculated from a single colony was grown in LB media under selective pressure (adding 40 µg/mL of kanamycin) at 37°C for 20-24 h. At time point zero, 10 mL of LB media without selective antibiotics were inoculated with the overnight culture to an OD_{600} of 0.2. An aliquot of the freshly inoculated liquid culture was diluted in LB media and the dilutions (10^{-6}-10^{-8}) were plated onto LB-agar plates without selective antibiotic. The remaining liquid culture was grown for 24 h and the plates were incubated at 37°C for 20-24 h. The resulting colonies were counted to determine the CFU and tested for antibiotic resistance by stamping plates with appropriate amount of colonies (10-30 colonies per plate) onto LB-agar plates containing 40 µg/mL of kanamycin as selective antibiotic, in order to determine the percentage of cells that had retained their plasmid. The stamped plates were incubated for 20-24 h. After 24, 48, 72 and 96 h the whole procedure starting with the inoculation of 10 mL LB media without selective antibiotics with the preceding culture was repeated.

In *Figure 21* a scheme for the plasmid stability assay of *E. coli* Top10 strains is illustrated.

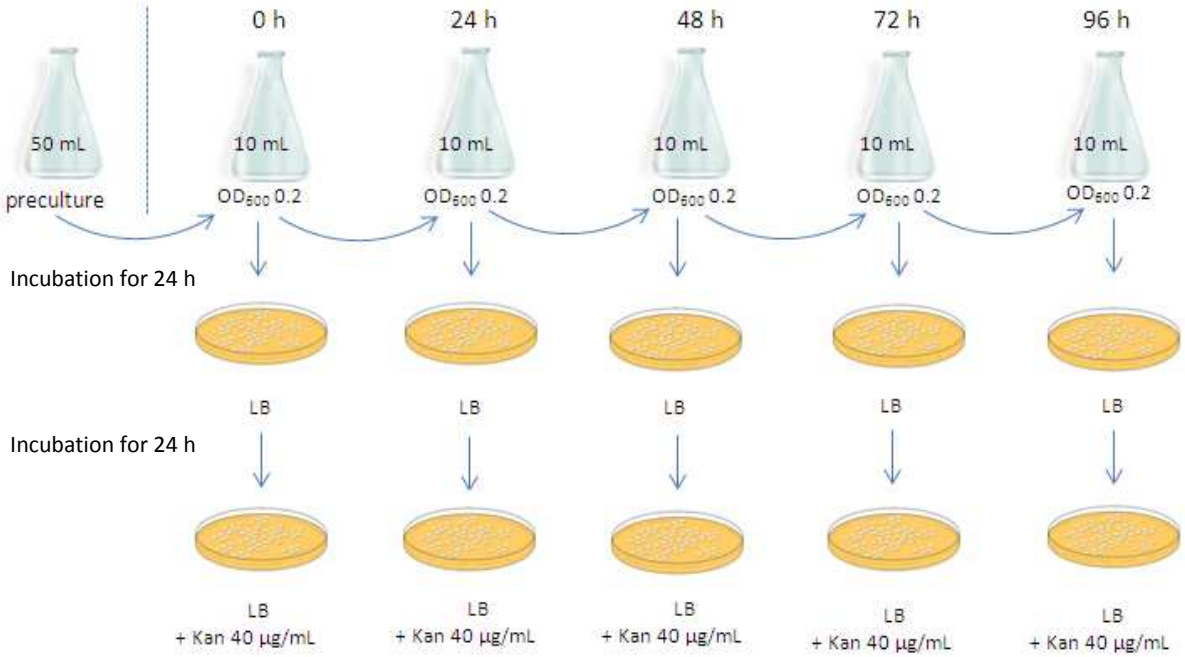


Figure 21: Scheme for the plasmid stability assay of E. coli Top10 strains: The preculture was inoculated from a singly colony in LB media with 40 µg/mL kanamycin and incubated at 37°C for 20-24 h. After inoculating the liquid culture at time 0 h with the preculture to an OD_{600} of 0.2 an aliquot of 100 µL was diluted (10^{-6}-10^{-8}) and plated on LB agar plates. The plates were incubated at 37°C for 24 h and the resulting colonies were counted to determine the CFU. By stamping plates with appropriate amount of colonies (10-30 colonies per plate) onto LB agar plates + 40 µg/mL kanamycin as selective antibiotic the percentage of cells that had retained their plasmid was determined. The whole procedure was repeated after 24, 48, 72 and 96 h.

For the *R. eutropha* H16 strains the procedure was similar. However, 20 µg/mL of gentamycin was added to the employed TSB media (TSB_{gen20}) and TSB agar plates (TSB_{gen20} agar plates) in order to minimize the contamination risk with other bacteria. For the selective pressure 200 µg/mL of kanamycin were added. The liquid overnight culture inoculated from a single colony was grown in TSB_{gen20} media under selective pressure (adding 200 µg/mL of kanamycin) at 28°C for 60-72 h. At time point zero, 10 mL of TSB_{gen20} media without selective antibiotics were inoculated with the overnight culture to an OD_{600} of 0.2. An aliquot of 100 µL from the freshly inoculated liquid culture was diluted in TSB_{gen20} media and the dilutions (10^{-5}-10^{-6}) were plated onto TSB_{gen20} agar plates without selective antibiotic. The remaining liquid culture was grown for 24 h and the plates were incubated at 28°C for 70-72 h. The resulting colonies were counted to determine the CFU and tested for antibiotic resistance, by stamping plates with appropriate amount of colonies (10-30 colonies per plate) onto TSB_{gen20} agar plates containing 200 µg/mL of kanamycin as selective antibiotic. The percentage of cells that had retained their plasmid was determined. The stamped plates were incu-

bated for 20-24h. After 24, 48, 72 and 96 h the whole procedure starting with the inoculation of 10 mL TSB_{gen20} media without selective antibiotics with the preceding culture was repeated.

In *Figure 22* a scheme for the plasmid stability assay of *R. eutropha* H16 strains is illustrated.

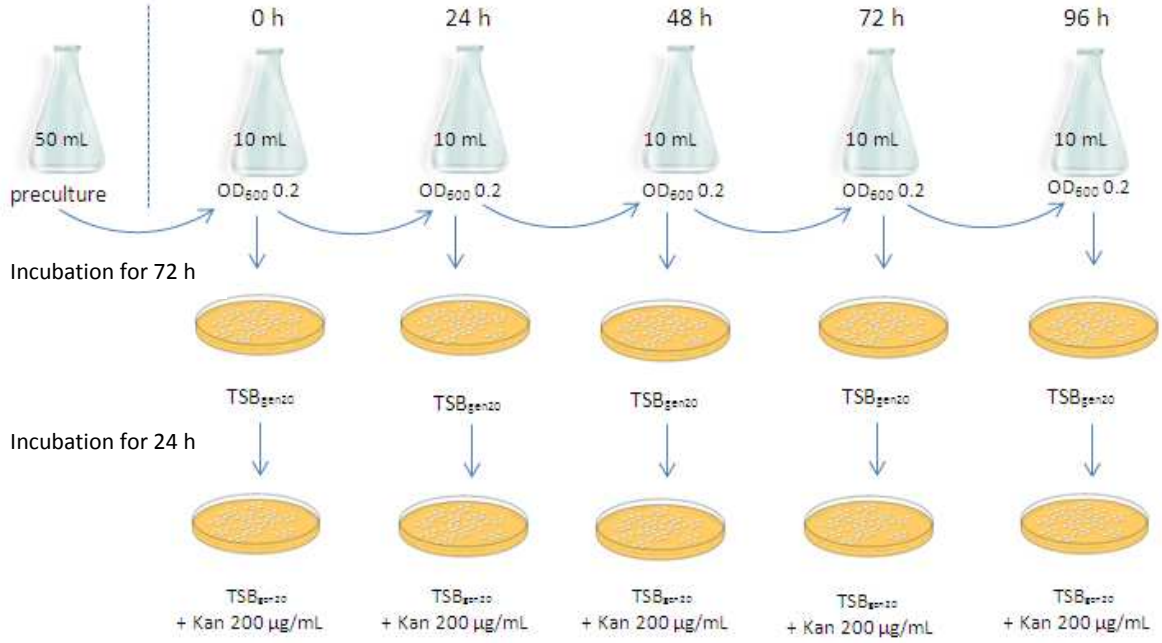


Figure 22: Scheme for the plasmid stability assay of R. eutropha H16 strains: The preculture was inoculated from a singly colony in TSB_{gen20} media with 200 µg/mL kanamycin and incubated at 28°C for 60-72 h. After inoculating the liquid culture at time 0 h with the preculture to an OD_{600} of 0.2 an aliquot of 100 µL was diluted (10^{-5}-10^{-6}) and plated on TSB_{gen20} agar plates. The plates were incubated at 28°C for 72 h and the resulting colonies were counted to determine the CFU. By stamping plates with appropriate amount of colonies (10-30 colonies per plate) onto TSB_{gen20} agar plates + 200 µg/mL kanamycin as selective antibiotic the percentage of cells that had retained their plasmid was determined. The whole procedure was repeated after 24, 48, 72 and 96 h.

In addition to the determination of the CFUs and the percentage of cells that had retained their plasmid, the average generation time and the number of generation in 24 h and in 96 h could be calculated via the measured OD_{600} values during the plasmid stability assay:

$$Number\ of\ \ generations\ in\ 24\ h = \frac{\log OD_{600}\ \ after\ 24\ h - \log OD_{600}\ \ at\ inoculation}{\log 2}$$

$$Number\ of\ \ generations\ in\ 96\ h = \frac{number\ of\ generations\ in\ 24\ h}{24\ h} * 96\ h$$

$$Generation\ time = \frac{24\ h}{number\ of\ \ generations\ in\ 24\ h}$$

2.6 Detection methods for eGFP

2.6.1 Fluorescence microscope

For the detection of fluorescence the Leica DM LB2 microscope (Wetzlar, Germany) with the HCX FL Fluotar 100x/1.30 oil objective in phase contrast (PH3) mode was used. Images were taken using a Leica DFC350 FX monochrome Digital Camera. EGFP fluorescence was detected via a Leica 100 W high pressure mercury lamp using emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue). All images were acquired and amplified eightfold using the Corel photo editor software.

For fluorescence microscopy the *R. eutropha* H16 strains were grown as liquid culture in 15 mL tubes, using 5 mL LB, MSM or TSB media with selective antibiotics for 60-72 h, until they reached an OD_{600} between 0.8-3.5. For microscopy 5-8 µL of the liquid culture were applied on a class plate (Carl Roth GmbH CO. KG) and covered with a covering glass (Carl Roth GmbH CO. KG).

2.6.2 SDS-PAGE

The SDS-PAGE gels were performed with electrophoresis equipment from Hoefer, Inc. (SE 250 Mighty Small II, Hoefer, Inc.; Holliston, USA). First the resolving gel was poured and covered with butanol. After 30 min the butanol was removed and the stacking gel was poured on the top of the resolving gel. After another 30 min the gel was ready to load with the samples. All components for the gel are listed in *Table 10* .

Table 10: Components for the stacking and resolving gel for the SDS-PAGE (given volumes for two gels).

Components	Stacking gel (4%)	Resolving gel (12%)
H_2O	5,6 mL	6 mL
Acrylamid (Bio-Rad, Hercules, USA)	6,4 mL	1,5 mL
Resolving gel buffer (0.5 M Tris, 0.4 % SDS, pH 8.8)	-	4 mL
Stacking gel buffer (0.5 M Tris, 0.4 % SDS, pH 6.8)	2.5 mL	-
APS (10%)	50 µL	20 µL
TEMED	20 µL	15 µL
Bromophenol Blue	-	10 µL

For sample preparation 1 mL of fresh liquid cell cultures with known OD_{600} were centrifuged and the supernatant was removed. The cell pellet was resuspended in potassium phosphate buffer (pH7.4, 0.1 M). The volume was calculated with the following formulas:

- For *E. coli* Top 10 strains: V_{buffer}= 33.3 * OD_{600}
- For *R. eutropha* H16 strains: V_{buffer}= 15 * OD_{600}

For *R. eutropha* strains 10-15 µL of the resuspended cells and for *E. coli* strains just 3 µL were used for further sample preparation:

R. eutropha	***E.coli***	
15 µL	3 µL	**Resuspended cells**
5 µL	5 µL	**FSB buffer (5x)**
2 µL	2 µL	**NaOH (0.1 M)**
2 µL	2 µL	**SDS (10%)**
1.2 µL	1.2 µL	**β-Mercaptoethanol**
-	12 µL	**K_3PO_4 buffer (pH7.4, 0.1 M)**
25.2 µL	25.2 µL	

The samples were heated at 99°C for 10 min in a thermomixer, briefly centrifuged and afterwards loaded directly on the gel. As standard 5 µL of the PageRuler™ Prestained Protein Ladder (Thermo Scientific, St. Leon-Rot, Germany) (see *Figure 23*) were applied. The gel was

covered with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and run for 10 min at 200 V and then for ~ 60 min at 110 V.

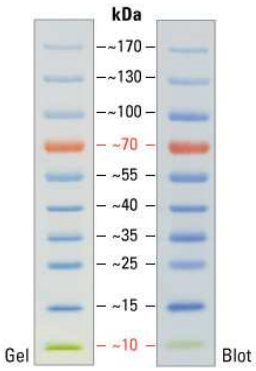


Figure 23: Standard for SDS-PAGE: PageRuler™ Prestained Protein Ladder (Thermo Scientific).

Afterwards the gel was stained in Comassie Brilliant Blue (Brilliant Blue G 250, CARL ROTH GMBH + CO. KG; Heidelberg, Germany) for 30 min and then destained again in 10% acetic acid overnight.

2.6.3 Western Blot

For the Western Blot the unstained SDS-PAGE gel was transferred onto a nitrocellulose membrane (Roti-NC HP40.1, 0.2 µm, Carl Roth GmbH + CO.KG) using the TE22 Mini transfer tank unit from Hoefer, Inc. (Holliston, USA). The transfer was performed at 240 mA for 45 min with the Power Ease500 from Invitrogen (Carlsbad, California, USA)

All necessary buffers during the blocking and washing procedure of the membrane are listed in *Table 11.*

Table 11: Buffers used for the Western Blot

buffer	component
PBS (10x)	80 g NaCl, 2 g KCl, 17.8 Na_2HPO_4 x $2H_2O$, 2.4 g KH_2PO_4, add to 1000 mL H_2O; pH 7.4
TBS	6.05 g Tris, 8.76 g NaCl, add to 1000 mL H_2O; pH 7.5
1x PBS – 0.05% Tween 20 (Washing buffer)	500 µL Tween 20, add to 1000 mL PBS (1x)
Transfer buffer (20x)	14.5 g Tris, 72 g glycine, add to 500 mL H_2O
Transfer buffer (1x)	50 mL transfer buffer (20x), 100 mL MeOH, add to 1000 mL H_2O

Afterwards the membrane was blocked with 1 % milk powder (dissolved in 50 mL of 1x PBS) overnight at 4°C. The primary mouse eGFP antibody (dissolved in 1x PBS with 1% BSA, 1:1000 dilution) [Mono-clonal Anti-Green Fluorescent protein (GFP), N-terminal, antibody produced in mouse, 2 mg/mL, G6795, Sigma-Aldrich; Vienna, Austria] was applied onto the membrane for 2 h. After washing the membrane four times for 5 min with 1x PBS – 0.05% Tween 20 buffer the secondary goat anti-mouse antibody with alkaline phosphates (dissolved in 50 mL 1% TBS and 1% BSA, 1:5000 dilution) (Goat-anti-mouse lgG-AP: Sc-2008, alkaline phosphatase conjugated, 400 µg/mL, Santa Cruz Biotechnology, Inc.; Santa Cruz, USA) was applied on the membrane for 1 h. Then the membrane was washed again four times for 5 min. For the detection of the alkaline phosphatase a BCIP/NBT solution mixture (Invitrogen; Carlsbad, California, USA) was applied onto the membrane until a color development was visible.

2.6.4 Measurement of Fluorescing Units

For the measurement of the fluorescing units the *R. eutropha* H16 strains were grown in liquid LB media or MSM-Media + 3% Fructose containing the appropriate antibiotics (*R. eutropha* H16: Gentamycin 20 µg/mL; *R. eutropha* H16 + plasmids: Gentamycin 20 µg/mL, Kanamycin 200 µg/mL). The OD_{600} of the liquid culture was normalized by using 4 mL of a culture with an OD_{600} of 2.

The normalized amount of the liquid culture was centrifuged (in 15 mL Greiner tube) for 5 min and 4000 rpm at 4°C. The supernatant was discarded, 500 µL B-Per (Thermo Scientifc) were added and the cell pellet was carefully resuspended (20 times up and down pipetting). The mixture was transferred in a 1.5 mL Eppendorf tube and centrifuged for 5 min at full speed at 4°C. The sample was kept on ice until measurement.

The fluorescing units were measured with a fluorimeter from BMG Labtech (FLUOstar Omega, BMG Labtech; Ortenberg, Germany). Therefore 200 µL of the supernatant were pipetted in the wells of a black microtiter plate. The used program (eGFP_Steffen) included a preshaking of the plate for 10 sec at 200 rpm. Excitation and emission wavelengths of 485 nm and 520 nm, respectively, were used. Every sample was measured twice.

2.7 Detection methods for LacZ

For the detection of LacZ two well-known methods were applied. After the transformation of the pKR-Tac-Par-LacZΔlacI REP vector into *E.coli* Top10 the cells were plated out on agar plates containing 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal). This sugar component is an analog of lactose and can be hydrolyzed enzymatically by β-galactosidase generating the 5-Bromo-4-chloro indigo dye which turns blue under the presence of oxygen. Via this blue-white screening positive clones of *E. coli* [pKR-Tac-Par-LacZΔlacI REP] could easily be identified.

However, this method cannot be applied for *R. eutropha* H16 strains because the lactose transport system which would be necessary for the assimilation of X-gal is missing [8], [88]. Therefore the β-galactosidase assay was employed for the detection of the presence of *lacZ* and the expression of β-galactosidase. This assay is a well-developed method to measure the level of β-galactosidase. The assay is based on the enzymatic conversion of the colorless ortho-Nitrophenyl-β-galactoside (oNPG) via the β-galactosidase into galactose and the yellow ortho-Nitropheol (oNP). The amount of produced oNP can be measured at 420 nm. For the assay the cells have to be grown in minimal medium A overnight at 37°C (*E. coli* Top10 strains) or 28°C (*R. eutropha* H16 strains). The next day the OD_{600} was measured in microtiter plates at the plate reader (FLUOstar Omega, BMG Labtech; Ortenberg, Germany). Then 150 µL of the ONCs were mixed with 850 µL Z-buffer (add 50 mM β-Mercaptoethanol just before the assay). For *E.coli* Top 10 strains 50 µL chloroform and 25 µL SDS (0.1%) were applied to the samples. For *R. eutropha* H16 strains the double amount of chloroform (100 mL) and SDS (50 µL) had to be added to achieve proper cell disruption [89]. Then the samples were mixed equally for around 30 sec (7-8 times up and down pipetting). After the mixing 125 µL of the upper phase were pipetted into a microtiter plate and incubated for 5 min at room temperature. Then the reaction was started via adding 25 µL of oNPG-solution for *E.coli* Top 10 strains and 200 µL oNPG to *R. eutropha* H16 strains [89]. After a defined incubation time (3 min for *E.coli* strains, 15-30 min for *R. eutropha* strains) the reaction was stopped by adding 65 µL Na_2CO_3 (1 M). All necessary buffers, media and solutions are listed in *Table 12.*

Table 12: Necessary buffers, media and solutions for the β-galactosidase assay

buffer/media/solutions	components
Z-buffer	10.7 g/L Na_2PHO_4, 6.3 g/L NaH_2PO_4, 10 mM KCl, 1 mM MgSO4, add to 1000 mL H_2O; pH 7; add 50 mM β-Mercaptoethanol
phosphate buffer (0.1 M)	2.75 g Na_2PHO_4, 1.46 g NaH_2PO_4 add to 250 mL H_2O; pH 7
Minimal media A	10.5 g/L K_2PHO_4, 4.5 g/L KH_2PO_4,, 1.0 g/L$(NH_4)_2SO_4$, 0.5 g/L Na-Citrat, 3.0 g/L Casamino acids (FIRMA) add to 977 mL H_2O, autoclave; add 1 mL $MgSO_4$ (1 M), 20 mL Glucose (20%), 2 mL Thiamin (10 mg/mL) (sterile filtrated)
oNPG (4 mg/mL)	dissolve in 0.1 m phosphate buffer

Afterwards the absorbance of the samples was measured at 420 nm (measuring the amount of produced oNP) and at 550 nm (measuring the absorbance and scattering caused by cell residues), using Z-buffer with oNPG solution and Na_2CO_3 as blank.

In order to determine and compare the produced amount of β-galactosidase the Miller-Units could be calculated:

$$\text{Miller-Units} = 1000 * \frac{OD_{420} - 1.75 * OD_{550}}{t * V * OD_{600}}$$

OD_{420}: amount of produced oNP

OD_{550}: scattering caused by cell residues

OD_{600}: optical cell density

t: time [min]

V: volume of used cell culture [mL]

3 Results

3.1 Vector construction

3.1.1 pKR-Tac-Par-eGFPΔlacI REP

The construction of the pKR-Tac-Par-eGFPΔlacI REP vector was based on the pKR-Tac-Par-29.1-eGFPΔlacI REP vector containing the Tac promoter, the REP origin of replication, the kanamycin resistance gene, *egfp* as reporter gene and the *par29.1* sequence originated from the pCM470 DsbC APLEC8P par29 plasmid. However, in *par29.1* the last 50 bp of the original *parE* and a terminator sequence were missing. Therefore the pKR-Tac-Par-29.1-eGFPΔlacI REP vector was cut with *Spe*I to replace the old *par29.1* sequence with the new *par* sequence from the RK2 plasmid. The *par* region was amplified via overlap extension PCR, in order to delete an *Nde*I restriction site in between the *par* sequence. Two standard PCRs (conditions see *Table 6*) were performed: from the beginning of the *parA* gene to the *Nde*I (1550 bp) site and from the *Nde*I site to the end of the *parE* gene (589 bp). The control gel of the two PCR products is shown in *Figure 24* (A). The two fragments were then joined via an overlap extension PCR (conditions see *Table 7*) resulting in the *par* sequence with a deleted *NdeI* site. Furthermore a terminator sequence ($T_{L13.2}$) was added to the end of *parE* and on both ends of the sequence *Spe*I restriction sites were attached. In *Figure 24* (B) the control gel of the overlap extension PCR product (2256 bp) (lane1) as well as the cut vector backbone of pKR-Tac-*par29.1*-eGFPΔlacI REP (4653 bp) (lane2) is shown.

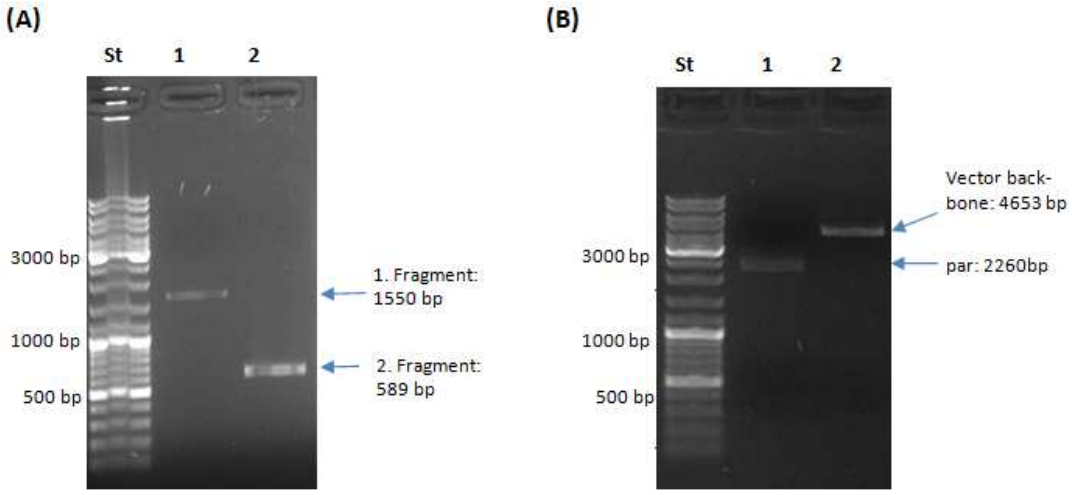


Figure 24: ***(A)*** *Agarose gel of the two PCR products used for the overlap extension PCR of the par sequence: St: GeneRuler™ DNA Ladder Mix 500 ng, lane 1: Fragment from parA to NdeI (1550 bp), lane 2: Fragment from NdeI to parE (589 bp);* ***(B)*** *Agarose gel of the overlap extension PCR product for the par sequence and of the vector backbone: St: GeneRuler™ DNA Ladder Mix 500 ng, lane 1: par sequence (2256 bp), lane 2: pKR-Tac-par29.1-egfp- ΔlacI cut with SpeI (4653 bp);*

After ligation of the vector backbone with the *par* sequence and transformation in *E. coli* Top10 green fluorescing colonies, showing the expression of eGFP, could be detected. Out of four clones plasmid DNA was isolated and a control cut with *Spe*I was performed and analyzed via gel electrophoresis. (See *Figure 25*)

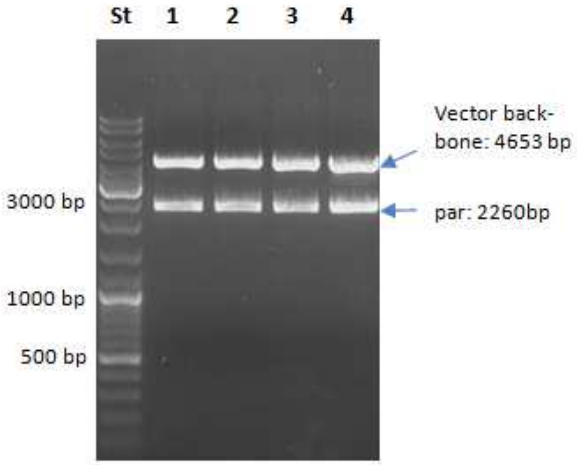


Figure 25: Agarose gel of the control cut of the isolated plasmid DNA out of four different clones (lane 1-4): pKR-Tac-Par-egfpΔlacI Rep cut with SpeI (vector backbone: 4653 bp, par Sequence: 2256 bp), St: GeneRuler™ DNA Ladder Mix 500 ng;

The isolated plasmid DNA of all clones showed the right restriction pattern for the vector backbone (at 4653 bp) and the par sequence (at 2256 bp). The plasmid DNA of the first clone

was transformed in *R. eutropha* H16 and used for further experiments like the plasmid stability assay and the detection of eGFP.

3.1.2 pKR-Tac-Par-eGFPΔlacI oriV393/oriV632

In order to establish a set of expression vectors with different origins of replication the REP sequence of the pKR-Tac-Par-eGFPΔlacI REP was replaced with two forms of the *oriV* sequence from the RK2 plasmid – oriV393 and oriV632. For further description of the *oriV* sequence see *Figure 2.*

For the construction of the pKR-Tac-Par-eGFPΔlacI oriV393 and the pKR-Tac-Par-eGFPΔlacI oriV 632 vectors the sequence of the short oriV393 and the longer oriV632 region from the RK2 plasmid were amplified via PCR. However, in order to remove a tetracycline resistance gene in between the *oriV* and the *Rep* sequence two standard PCRs were performed (conditions see *Table 6*). Both sequences were amplified with primers containing an additional overlap of 20 bp. The control gel of the three PCR products is shown in *Figure 26* (A). The particular fragments – Rep and *oriV* with 393 bp or Rep with *oriV* with 632 bp – were then combined via an overlap extension PCR (conditions see *Table 7*) resulting in the oriV393 (2097 bp) or the oriV632 (2335 bp) (see *Figure 26* (B)).

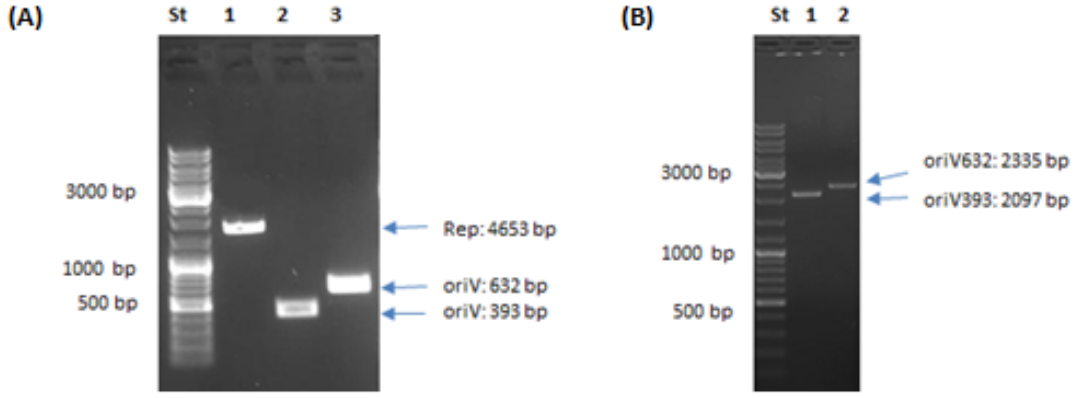


Figure 26: ***(A)*** *Agarose gel of the PCR products of the overlap extension PCR for the oriV393/oriV632: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: Rep sequence originated from RK2 plasmid (1618 bp) and lane 2: 393 bp fragment of oriV from RK2 plasmid (393 bp), lane 3:632 bp fragment of oriV from the RK2 plasmid ;* ***(B)*** *Agarose gel of the overlap extension PCR for the oriV393 and oriV632: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: oriV393 (2097 bp) and lane 2: oriV632 (2335 bp);*

The exchange of REP seqeunce in the pKR-Tac-Par-eGFPΔlacI REP vector with the oriV393 or oriV632 via the *SpeI* and *PstI* restriction sites included the removal of the *par* sequence

which resulted in an unstable vector. The *par* sequence had to be moved in another position before the new origins could be introduced. Therefore the pKR-Tac-Par29.1-eGFP REP vector, containing the *lac*I gene between the *Pst*I and *Not*I restriction was used. The old *par29.1* was removed via cutting with *Spe*I and the vector backbone was relegated afterwards. Then the *lac*I gene was removed via cutting with *Pst*I and *Not*I and replaced with the *par* sequence, which was amplified via PCR with new primers attaching a *Pst*I and *Not*I restriction site to the end of *parA* and of *parE* gene. The control gels for the vector backbone (pKR-Tac-ΔPar29.1-eGFPΔlacI, 4107 bp) and the new *par* sequence with *Pst*I and *Not*I restriction site (2660bp) are shown in *Figure 27* (A) and (B). After the ligation and transformation of the new designed vector pKR-Tac-Par-eGFPΔlacI Rep with the *par* sequence between the *Pst*I and *Not*I restriction sites in *E.coli* Top10 cells the vector was isolated for a control cut. The control gel is shown in *Figure 27* (C).

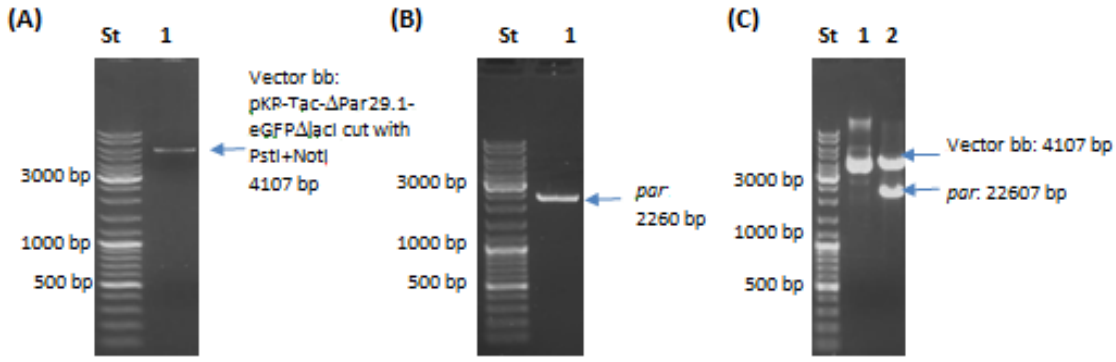


Figure 27: ***(A)*** *Agarose gel of the vector backbone: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: pKR-Tac-ΔPar29.1-egfpΔlacI cut with PstI+NotI (4107 bp);* ***(B)*** *Agarose gel of the par PCR product with PstI and NotI restriction site: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: par sequence with PstI+NotI (2260 bp);* ***(C)*** *Agarose gel of control cut of the new designed vector pKR-Tac-Par-egfpΔlacI Rep with PstI+NotI: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: uncut vector, lane 2. Cut vector with PstI and NotI (vector backbone: 4107 bp, Par: 2260 bp).*

The new vector pKR-Tac-Par-eGFPΔlacI REP with the *par* sequence located between *Pst*I and *Not*I was cut with *Spe*I and *Pst*I in order to exchange the old origin of replication REP with the new designed oriV393 or oriV632. After the ligation of the backbone (4652 bp) with the new origins (oriV393: 2097 bp, oriV632: 2335 bp) and the transformation of the new vectors in *E. coli* Top10 the grown colonies showed a strong fluorescence signal under see microscope, indicating the successful expression of eGFP. The plasmid DNA was isolated from the gained clones (10 clones per origin) and cut with *Spe*I and *Pst*I to determine the positive ones. The

control cuts of the plasmid DNA of the different clones for the vector pKR-Tac-Par-eGFPΔlacI oriV393 respectively oriV632 are shown in *Figure 28* (A) and (B).

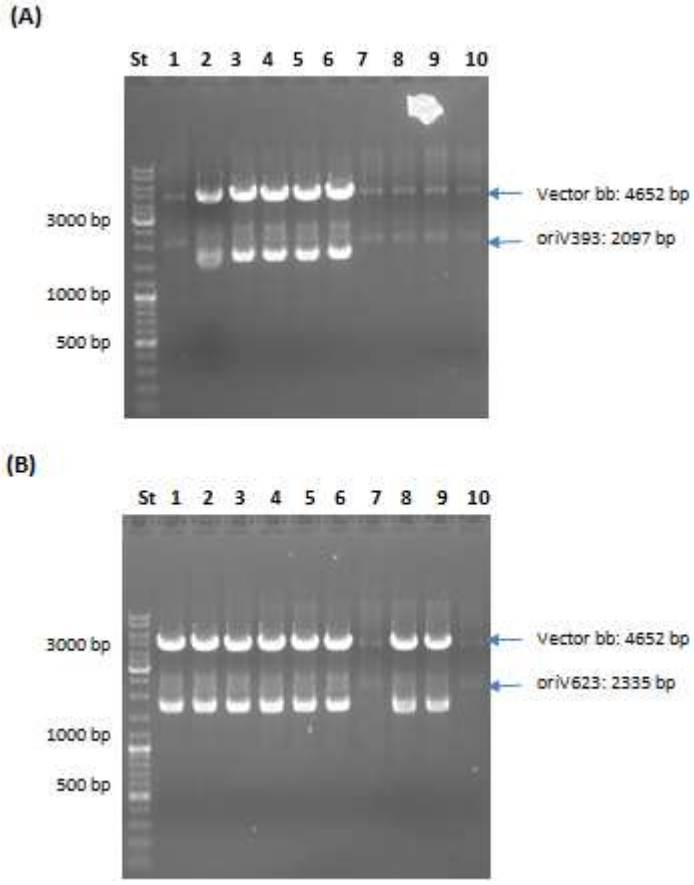


Figure 28: ***(A)*** *Agarose gel of the control cut of the pKR-Tac-Par-egfpΔlacI oriV393 vector: St. GeneRuler™ DNA ladder Mix 500 ng, lane 1-10: cut vector DNA of the clones 1-10 (appropriate bands: vector backbone: 4652 bp, oriV393: 2097 bp);* ***(B)*** *Agarose gel of the control cut of the pKR-Tac-Par-egfpΔlacI oriV632 vector: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1-10: cut vector DNA of the clones 1-10 (appropriate bands: vector backbone: 4652bp , oriV632: 2335 bp);*

For pKR-Tac-Par-eGFPΔlacI oriV393 the positive clones containing the desired plasmid were clones number 1, 7, 8, 9 and 10 (*Figure 28* (A) lane 1, 7, 8, 9, 10) and for pKR-Tac-Par-eGFPΔlacI oriV632 clones number 7 and 10 (*Figure 28* (B) lane 7, 10). Both clones number 7 were sent for sequencing. However, the sequencing results showed mutations in the region of the *oriV* – at the short and long variant. Therefore also the clones' number 10 and the pJET-vectors containing the oriV393 or oriV632 sequence were sequenced, which showed all similar mutations. Through comparison of both sequences to the original NCBI sequence of the Birmingham IncP α RK2 plasmid (accession number: BN000925) [30] two mutations could be located in the sequence of the smaller variant of the *oriV* (393 bp) and three muta-

tions in the longer form (632 bp). In *Table 13* the position of the mutations in the actual sequence compared to the NCBI sequence are shown.

Table 13: Mutations in the sequence of the oriV393 and oriV632 origin of replications compared to the sequence of the Birmingham IncP α RK2 plasmid [30].

Origin	Position	Mutation
oriV393	180 bp	A → C
	347 bp	additional T
oriV632	136 bp	A →T
	418 bp	A → C
	585 bp	additional T

The two mutations in the oriV393 sequence at 180 bp and 347 bp are the same mutations than in the oriV632 sequence at position 418 and 585. In the first mismatch at position 180 (at oriV393) and 418 (at oriV632), respectively an A to C exchange occurred. The second mutation led to an insertion of an additional tyrosine base at position 347 (at oriV393) and 585 (at oriV632). The oriV632 sequence is 293 bp longer than the oriV393 sequence and in this region also a third mutation, the exchange from an adenosine to a tyrosine base at position 136 occurred. However, the mutations are located in no specific regions of the *oriV* sequence. The two mutations occurring in both sequences are between the interon repeats and after the AT-rich region. The additional mutation on the oriV632 sequence is located around 20 bp upstream o the first iterons sequence. The sequence of the oriV393 and the oriV632 with the mutations (marked with red letters) is illustrated in Appendix 3: Sequences.

However, through Blast search several plasmid derived from the RK2 plasmid carrying the RK2 mini-replicon could be discovered, which possessed the same mutations found in our sequence. Some example for these plasmid are the pBS228 plasmid (accession number: AY204475) [90] as well as the pRK290 plasmid [91]. From the latter several different cloning vectors were derived like the pRK310 (Accession number: AF327712) [92] and the smaller pRK404 plasmid (Accession number: AY204475) [93] which are able to replicate in many gram-negative bacteria, like *R. eutropha* [94–96]. Therefore the differences in the *oriV* sequence of our vectors compared to the original RK2 sequence should have no effect on the function of the mini-replicon. The results of the Blast search may lead to the assumption that two different, but functional, versions of the *oriV* sequence of the RK2 plasmid exist.

For the preceding experiments regarding the plasmid stability tests and detection of eGFP the plasmid DNA of both vectors pKR-Tac-Par-eGFPΔlacI oriV393 and pKR-Tac-Par-eGFPΔlacI oriV632 was transformed into *R. eutropha* H16.

3.1.3 pKR-Tac-Par-eGFPΔlacI RSF1010

In order to establish a fourth expression vector with a different origin of replication the pKR-Tac-Par-eGFPΔlacI oriV393 vector was cut with *Spe*I and *Pst*I to replace the oriV393 with the RSF1010 from the pKT231 plasmid. The sequence of the RSF1010 origin of replication was amplified via PCR adding the restriction sites *Spe*I and *Pst*I. In *Figure 29* (A) and (B) the control gels of the RSF1010 PCR product (5746 bp) and the pKR-Tac-Par-eGFPΔlacI ΔoriV393 vector backbone (4652 bp) are shown.

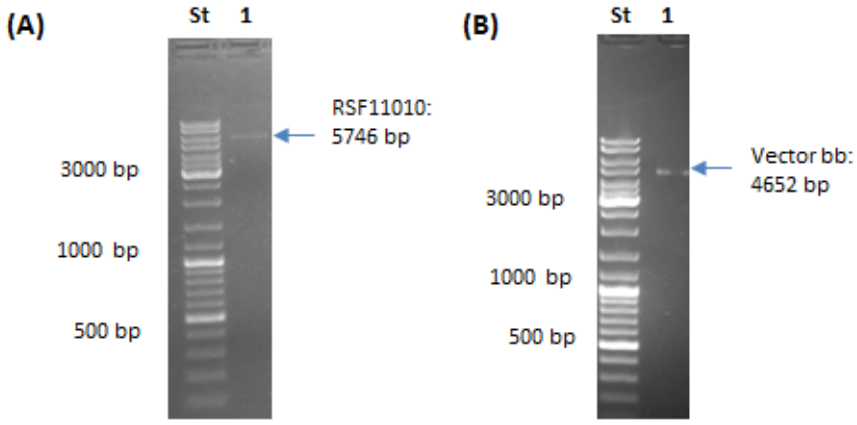


Figure 29: ***(A)*** *Agarose gel of the RSF1010 PCR product: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: RSF1010 (5746 bp);* ***(B)*** *agarose gel of the vector backbone of the pKR-Tac-Par-egfpΔlacI oriV393 cut with SpeI and PstI: St. GeneRuler™ DNA Ladder Mix 500 ng, lane1: vector backbone (4652 bp);*

After ligation and transformation of the new designed vector pKR-Tac-Par-eGFPΔlacI RSF1010 in *E.coli* Top10 cells green fluorescing transformants were streaked out for plasmid isolation and the plasmid DNA was analyzed via various restriction digests. In *Figure 30* the plasmid DNA from one clone was cut with the following enzyme combinations: *Spe*I (lane2, linearized vector: 10390 bp), *Pst*I (lane 3, linearized vector: 10390 bp), *Pst*I+*Not*I (lane 4, backbone: 8130 bp, Par: 2260 bp), *Spe*I+*Not*I (lane 5, backbone: 2404 bp, RSF1010+Par: 7986 bp), *Sma*I (lane 6, backbone: 8793 bp, eGFP+Kan^R: 1597 bp).

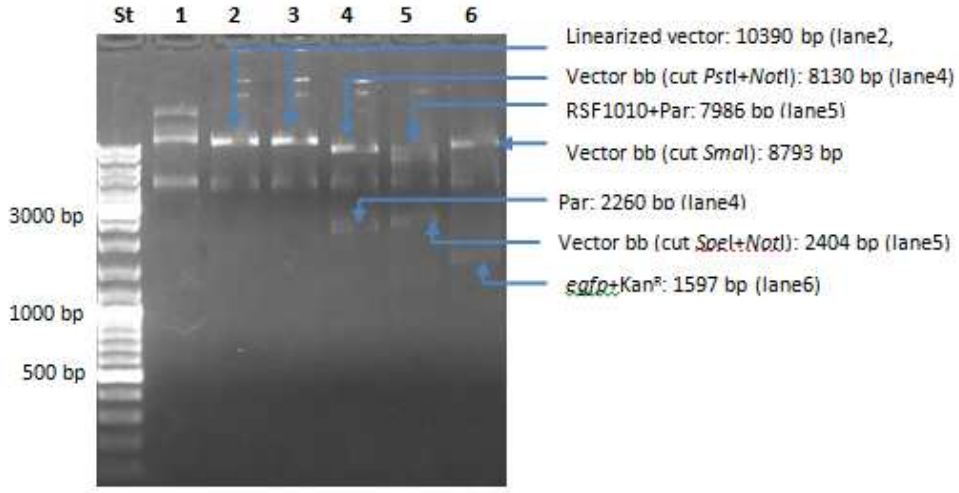


Figure 30: Agarose gel wih different control cuts of the pKR-Tac-Par-egfpΔlacI RSF1010 vector: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: uncut vector, lane 2: vector cut with SpeI (linearized vector: 10390 bp), lane 3: vector cut with PstI (linearized vector: 10390 bp), lane 4: vector cut with PstI+NotI (backbone: 8130 bp, Par: 2260 bp), lane 5: vector cut with SpeI and NotI (backbone: 2404 bp, RSF1010+Par: 7986 bp), lane 6: vector cut with SmaI (backbone: 8793 bp, egfp+Kan^R: 1597 bp);

The results of the enzyme digests of the pKR-Tac-Par-eGFPΔlacI RSF1010 (shown in *Figure 30*) showed the expected restriction patterns for all performed digests. However, for each restriction an additional band between 4000 to 5000 bp occurred. Regarding the uncut plasmid in lane 1 three or even four bands could be detected. The highest band could be the open circular form which is more bulky and moves rather slowly through the agarose gel, whereas the two bands at around 10000 bp should be the linear form of the plasmid. The lowest band between 4000 to 5000 bp could be a tight supercoiled form of the plasmid which is smaller and moves faster through the agarose gel then the linear form. Therefore, it seemed that the additional band at 4000 to 5000 bp occurring for each restriction digest is a very tight supercoiled form of the plasmid which could not be cut by the restriction enzymes. In order to confirm this assumption the band was cut out of the gel, cleaned up and transformed again in *E. coli* Top10 cells. The green fluorescence of the gained transformants was a signal for eGFP expression and the presence of the pKR-Tac-Par-eGFPΔlacI RSF1010 vector. The plasmid DNA of some clones was then again isolated and digested, resulting in the desired restriction pattern and the additional band at 4000-5000 bp.

Afterwards the isolated plasmid DNA of the analyzed clone was sent for sequencing. As no mutation in the RSF1010 sequence was found the pKR-Tac-Par-eGFPΔlacI RSF1010 vector

was transformed into *R. eutropha* H16 via electro transformation and conjugation. Further experiments regarding plasmid stability tests and detection of eGFP were performed.

3.1.4 pKR-Tac-Par-lacZΔlacI REP

In order to generate an expression vector containing another reporter gene the *egfp* gene in the pKR-Tac-Par-eGFPΔlacI REP vector was replaced with the *lacZ* gene. *LacZ* was cut out of the pRS415 using. *egfp* was removed from the pKR-Tac-Par-eGFPΔlacI REP vector by cutting with the *Xba*I and *Hind*III restriction enzymes and the backbone (5606 bp) was ligated with *lacZ* (3078 bp) and transformed into *E. coli* Top10. The control gel of the vector backbone and insert is shown in *Figure 31* (A). After the ligation and the transformation in *E. coli* the cells were plated on X-Gal plates in order to detect positive transformants via the blue/white screening. The new vector was isolated from the blue clones and cut with *Xba*I and *Hind*III to determine the positive ones. The control cuts of the isolated plasmid DNA of nine different clones are shown in *Figure 31* (B).

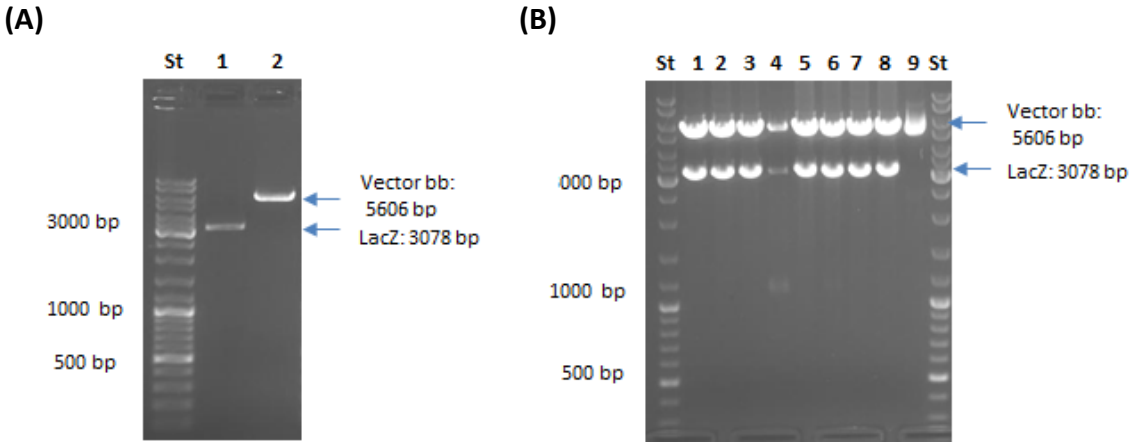


*Figure 31: **(A)** Agarose gel of the lacZ gene from the pRS415 and the vector backbone of pKR-Tac-Par-egfpΔlacI Rep, both cut with XbaI and HindIII: St. GeneRuler™ DNA Ladder Mix 500 ng, lane1: lacZ cut with XbaI and HinIII (3078 bp), lane 3: pKR-Tac-Par-egfp ΔlacI Rep cut with XbaI and HindIII (5606 bp); **(B)** Agarose gel of the control cut of the pKR-Tac-Par- lacZΔlacI Rep vector with XbaI and HindIII: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1-9: clones 1-9 (appropriate bands: vector backbone: 5606 bp, lacZ: 3078 bp)*

After the restriction analysis the pKR-Tac-Par- LacZΔlacI REP vector from clone number 7 was transformed into *R. eutropha* H16 and tested for the expression of the β-galactosidase.

However, via cutting the pKR-Tac-Par-eGFPΔlacI REP vector with *Xba*I the Shine-Dalgarno sequence, which is located between the *Xba*I and the *Nde*I restriction site, was removed

from the vector. In order to maintain the Shine Dalgarno sequence on the vector ensuring smooth expression, the *lacZ* was amplified via PCR from the pRS415, adding an *Nde*I and *Hind*III restriction site. Due to an *NdeI* restriction site, which was located on the end of the *lacZ* sequence (marked in *Figure 17*) an overlap extension PCR was necessary for its mutation. First two standard PCRs were performed from the beginning of *lacZ* to the *NdeI* (~3000 bp) site and from *NdeI* site to the end of *lacZ* (~100 bp). The concentration of the PCR products was measured via the NanoDrop2000 (Thermo Scientifc). The two fragments were then combined via an overlap extension PCR resulting in *lacZ* with a deleted *NdeI* site (3085 bp). After cutting the *lacZ* PCR product and the pKR-Tac-Par-eGFPΔlacI REP vector with *Nde*I and *Hind*III the vector backbone (5650 bp) and the insert (3085 bp) for the ligation was received (see *Figure 32 (A)*). After ligation and transformation of the new designed vector pKR-Tac-Par-LacZΔlacI REP in *E.coli* Top10 the cells were plated out on X-Gal plates to identfify positive transformants via the blue/white screening. Blue colonies were streaked out for plasmid isolation and the isolated plasmid DNA was tested via the control cut with *Nde*I and *Hind*III (see *Figure 32* (B)).

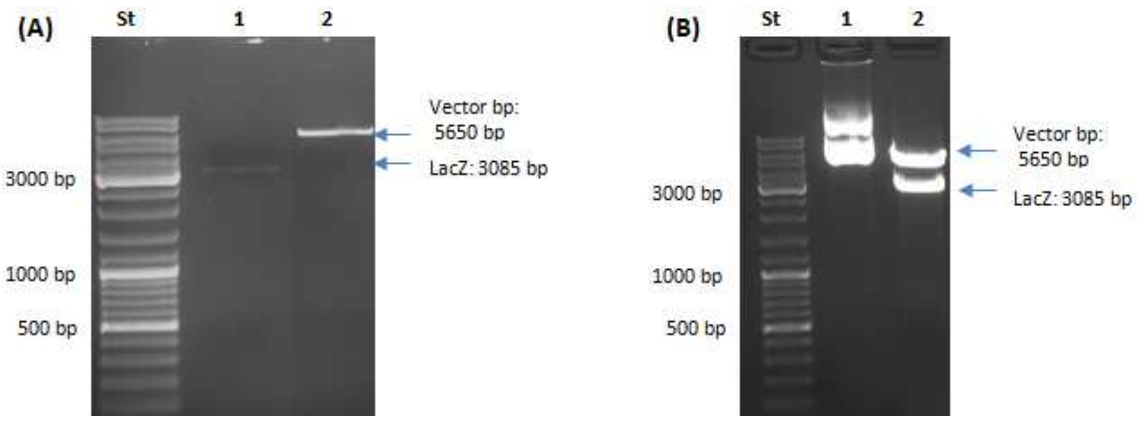


Figure 32: ***(A)*** *Agarose gel of the lacZ and the vector backbone of pKR-Tac-Par-egfpΔlacI Rep, both cut with NdeI and HindIII: St. GeneRuler™ DNA Ladder Mix, lane1: lacZ (3085 bp), lane 2: pKR-Tac-Par-egfpΔlacI Rep cut with NdeI and HindIII (vector backbone: 5650 bp);* ***(B)*** *Agarose gel of the control cut of the pKR-Tac-Par-lacZΔlacI REP vector with NdeI and HindII: St. GeneRuler™ DNA Ladder Mix, lane1: uncut vector, lane 2: pKR-Tac-Par-lacZΔlacI Rep cut with NdeI and HindIII (vector backbone: 5650 bp, lacZ 3085 bp);*

The plasmid DNA of the analyzed clone showed the right bands for the vector backbone (at 5650 bp) and the *lacZ* gene (at 3085 bp). Afterwards the DNA was send for sequencing. Hereby no additional mutations in the *lacZ* sequence were found and the pKR-Tac-Par-LacZΔlacI REP vector was directly transformed in *R. eutropha* H16 for the detection of the LacZ protein.

3.2 Plasmid stability

In the new designed pKR-Tac vectors the p*ar* region originating from the broad-host-range RK2 plasmid was introduced to enhance the distribution of the plasmids during cell division and to prevent plasmid loss. All vectors containing different origins of replication were transformed into *E.coli* Top10 and *R. eutropha* H16 and the plasmid stability was analyzed over a time period of 96 h.

The preculture was inoculated from a singly colony in appropriate media with selective antibiotic. In the following five days, each time after 24 h of incubation 10 mL of fresh media without selective antibiotics were inoculated with the corresponding preculture to an OD_{600} of 0.2. Out of this new culture an aliquot of 100 µL was used to prepare suitable dilutions for plating out on agar plates, which were incubated for 1 day at 37°C. For the *E.coli* Top 10 cultures the best dilutions were in the range of 10^{-5}-10^{-7}. The *R. eutropha* H16 cultures were diluted in the range of 10^{-5}-10^{-6} and plated out on TSB_{gen20} agar plates, which were incubated for 3 days at 28°C. After determining the CFUs plates containing an appropriate number of colonies (10-30 colonies per plate) were stamped onto agar plates containing a selective antibiotic and incubated for 24 h. By comparing the number of colonies in the agar plates with and without selective antibiotic the percentage of cells that had retained their plasmid was identified. Furthermore the number of generations in 24 h and in 96 h as well as the generation time per hour could be calculated. In the following tables the results for the different vectors in *E. coli* Top10 and *R. eutropha* H16 are listed.

In *Table 14* and *Table 15* the plasmid stability assay results of the plasmid pKR-Tac-Par-eGFPΔlacI REP in *E. coli* Top 10 and *R. eutropha* H16 are shown.

Table 14: Results of the plasmid stability assay of the E. coli Top 10 [pKR-Tac-Par-egfpΔlacI REP] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	1.57 [a]	1.98	1.82	3.52	3.92
Generation time [h]	-	7.2	7.3	6.0	5.6
number of generations in 24 h	-	3.3	3.2	4.0	4.3
CFUs on LB (1)	$5*10^7$	$6*10^7$	$7*10^6$	$8*10^7$	$9*10^6$
CFUs on LB + Kan 40µg/mL (1)	$5*10^7$	$6*10^7$	$7*10^6$	$8*10^7$	$9*10^6$
CFUs on LB (2)	$3*10^7$	$3*10^7$	$17*10^6$	$7*10^7$	$9*10^6$
CFUs on LB + Kan 40µg/mL (2)	$3*10^7$	$3*10^7$	$17*10^6$	$7*10^7$	$9*10^6$
CFUs on LB (3)	$2*10^7$	$6*10^6$	$2*10^7$	$3*10^7$	$8*10^6$
CFUs on LB + Kan 40µg/mL (3)	$2*10^7$	$6*10^6$	$2*10^7$	$3*10^7$	$8*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**6.4**		
Average no. of generations in 24 h			**3.75**		
Average no. of generations in 96 h			**15**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Table 15: Results of the plasmid stability assay of the R. eutropha H16 [pKR-Tac-Par-egfpΔlacI REP] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	3.23 [a]	2.51	3.54	3.82	4.36
Generation time [h]	-	6.6	6.0	5.7	5.4
number of generations in 24 h	-	3.6	4.0	4.2	4.4
CFUs on TSB + Gen 20 µg/mL (1)	$9*10^6$	$3*10^6$	$14*10^6$	$4*10^6$	$5*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (1)	$9*10^6$	$3*10^6$	$14*10^6$	$4*10^6$	$5*10^6$
CFUs on TSB + Gen 20 µg/mL (2)	$3*10^6$	$4*10^6$	$10*10^6$	$4*10^6$	$8*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (2)	$3*10^6$	$4*10^6$	$10*10^6$	$4*10^6$	$8*10^6$
CFUs on TSB + Gen 20 µg/mL (3)	$5*10^6$	$3*10^6$	$5*10^6$	$3*10^6$	$6*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (3)	$5*10^6$	$3*10^6$	$5*10^6$	$3*10^6$	$6*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**5.9**		
Average no. of generations in 24 h			**4.1**		
Average no. of generations in 96 h			**16**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Regarding the results of the plasmid stability it is obviously that the pKR-Tac-Par-eGFPΔlacI REP vector was 100% stable in *E. coli* Top 10 as well as in *R. eutropha* H16 over the time pe-

riod of 96 h. The average generation time in *E.coli* Top10 and in *R. eutropha* H16 was determined as 6.4 h respectively 5.9 h. Therefore the stability of the vector during 15 generations in *E. coli* Top 10 and during 16 generations in *R. eutropha* H16 had been proven. In *Figure 33* the stability of the plasmid in both strains is illustrated for the time period of 96 h.

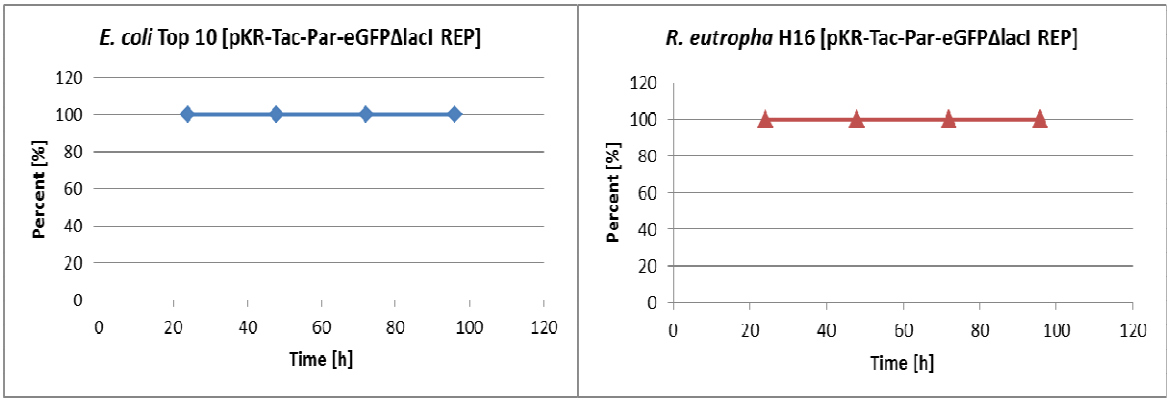


Figure 33: Stability of the plasmid pKR-Tac-Par-egfpΔlacI REP in E.coli Top10 and R. eutropha H16 over the time period of 96 h.

In *Table 16* and *Table 17* the results of the plasmid stability assay for strains *E. coli* Top10 [pKR-Tac-Par-eGFPΔlacI oriV393] and *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] are shown.

Table 16: Results of the plasmid stability assay of the E. coli Top 10 [pKR-Tac-Par-egfpΔlacI oriV393] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	1.85 [a]	2.98	2.75	2.83	2.43
Generation time [h]	-	6.0	6.3	6.0	6.7
number of generations in 24 h	-	4.0	3.8	4.0	3.6
CFUs on LB (1)	$32*10^8$	$8*10^6$	$33*10^6$	$10*10^5$	$12*10^6$
CFUs on LB + Kan 40µg/mL (1)	$32*10^8$	$8*10^6$	$33*10^6$	$10*10^5$	$12*10^6$
CFUs on LB (2)	$21*10^7$	$10*10^6$	$16*10^6$	$11*10^5$	$31*10^6$
CFUs on LB + Kan 40µg/mL (2)	$21*10^7$	$10*10^6$	$16*10^6$	$11*10^5$	$31*10^6$
CFUs on LB (3)	$40*10^7$	$20*10^6$	$31*10^6$	$24*10^5$	$9*10^6$
CFUs on LB + Kan 40µg/mL (3)	$40*10^7$	$20*10^6$	$31*10^6$	$24*10^5$	$9*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**6.3**		
Average no. of generations in 24 h			**4.0**		
Average no. of generations in 96 h			**16**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Table 17: Results of the plasmid stability assay of the R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV393] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	1.49 [q]	3.50	4.52	5.51	5.17
Generation time [h]	-	5.9	5.3	5.0	5.1
number of generations in 24 h	-	4.1	4.5	4.8	4.7
CFUs on TSB + Gen 20 µg/mL (1)	$11*10^6$	$9*10^5$	$11*10^6$	$40*10^5$	$50*10^5$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (1)	$11*10^6$	$9*10^5$	$11*10^6$	$40*10^5$	$50*10^5$
CFUs on TSB + Gen 20 µg/mL (2)	$92*10^5$	$8*10^5$	$8*10^6$	$32*10^5$	$52*10^5$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (2)	$92*10^5$	$8*10^5$	$8*10^6$	$32*10^5$	$52*10^5$
CFUs on TSB + Gen 20 µg/mL (3)	$70*10^5$	$21*10^5$	$6*10^6$	$50*10^5$	$22*10^5$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (3)	$70*10^5$	$21*10^5$	$6*10^6$	$50*10^5$	$22*10^5$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**5.3**		
Average no. of generations in 24 h			**4.5**		
Average no. of generations in 96 h			**18**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

The results of the plasmid stability assay showed 100% stability for the plasmid pKR-Tac-Par-eGFPΔlacI oriV393 in *E. coli* Top 10 as well as in *R. eutropha* H16 over the time period of 96 h. The average generation time in *E.coli* Top10 and in *R. eutropha* H16 was determined as 6.3 h and 4.5 h, respectively. The vector was 100% stable during 16 generation in *E.coli* Top10 and 18 generations in *R. eutropha* H16. In *Figure 34* the stability of the plasmid in both strains is illustrated for the time period of 96 h.

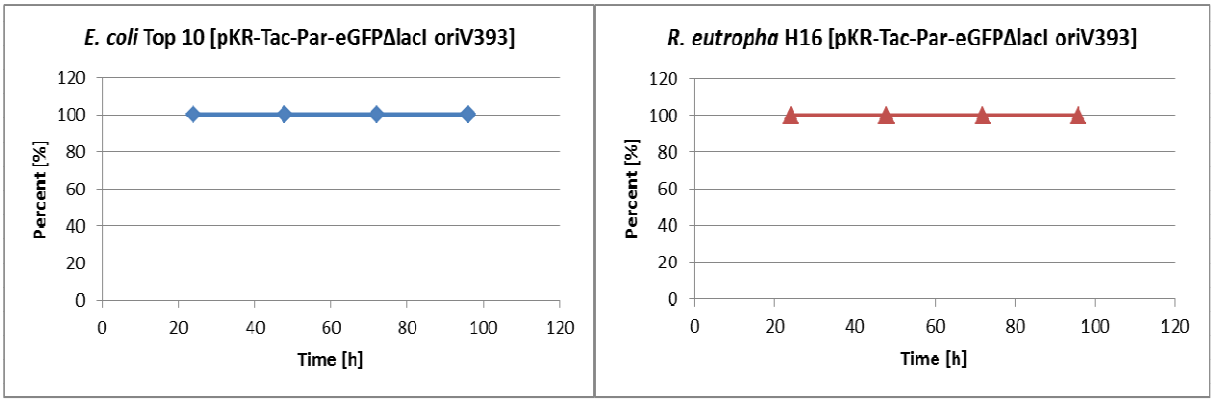


Figure 34: Stability of the plasmid pKR-Tac-Par-egfpΔlacI oriV393 in E.coli Top10 and R. eutropha H16 over the time period of 96 h.

The results of plasmid stability assay for the plasmid pKR-Tac-Par-eGFPΔlacI oriV632 in *E. coli* Top10 and *R. eutropha* H16 are shown in *Table 18* and *a Here* the OD600 of the preculture is given, which was inoculated with a single colony.

(1)-(3) mark the results of the triple determination.

Table 19.

Table 18: Results of the plasmid stability assay of the E. coli Top 10 [pKR-Tac-Par-egfpΔlacI oriV632] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	**24 h**	**48 h**	**72 h**	**96 h**
MW OD_{600} (precultures)	1.81 [a]	2.95	2.64	2.61	2.43
Generation time [h]	-	6.0	6.3	6.4	6.7
number of generations in 24 h	-	4.0	3.8	3.75	3.6
CFUs on LB (1)	$15*10^6$	$15*10^6$	$21*10^6$	$18*10^6$	$15*10^6$
CFUs on LB + Kan 40µg/mL (1)	$15*10^6$	$15*10^6$	$21*10^6$	$18*10^6$	$15*10^6$
CFUs on LB (2)	$39*10^6$	$28*10^6$	$16*10^6$	$16*10^6$	$10*10^6$
CFUs on LB + Kan 40µg/mL (2)	$39*10^6$	$28*10^6$	$16*10^6$	$16*10^6$	$10*10^6$
CFUs on LB (3)	$27*10^6$	$25*10^6$	$12*10^6$	$21*10^6$	$19*10^6$
CFUs on LB + Kan 40µg/mL (3)	$27*10^6$	$25*10^6$	$12*10^6$	$21*10^6$	$19*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**6.4**		
Average no. of generations in 24 h			**3.75**		
Average no. of generations in 96 h			**15**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Table 19: Results of the plasmid stability assay of the R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV632] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	1.76 [a]	3.18	3.31	3.89	4.26
Generation time [h]	-	6	6	5.6	5.4
number of generations in 24 h	-	4	4	4.3	4.4
CFUs on TSB + Gen 20 µg/mL (1)	$4*10^6$	$4*10^6$	$10*10^6$	$5*10^6$	$10*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (1)	$4*10^6$	$4*10^6$	$10*10^6$	$5*10^6$	$10*10^6$
CFUs on TSB + Gen 20 µg/mL (2)	$5*10^6$	$3*10^6$	$11*10^6$	$4*10^6$	$13*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (2)	$5*10^6$	$3*10^6$	$11*10^6$	$4*10^6$	$13*10^6$
CFUs on TSB + Gen 20 µg/mL (3)	$6*10^6$	$5*10^6$	$9*10^6$	$7*10^6$	$4*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (3)	$6*10^6$	$5*10^6$	$9*10^6$	$7*10^6$	$4*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**5.75**		
Average no. of generations in 24 h			**4.2**		
Average no. of generations in 96 h			**17**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

The pKR-Tac-Par-eGFPΔlacI oriV632 vector showed 100 % stability in *E. coli* Top 10 as well as in *R. eutropha* H16 over the time period of 96 h .(See *Figure 35*) The average generation time in *E.coli* Top10 and in *R. eutropha* H16 was determined as 6.4 h respectively 5.75 h. Therefore the vector was 100% stable during 15 and 17 generation in *E.coli* Top10 and in *R. eutropha* H16, respectively.

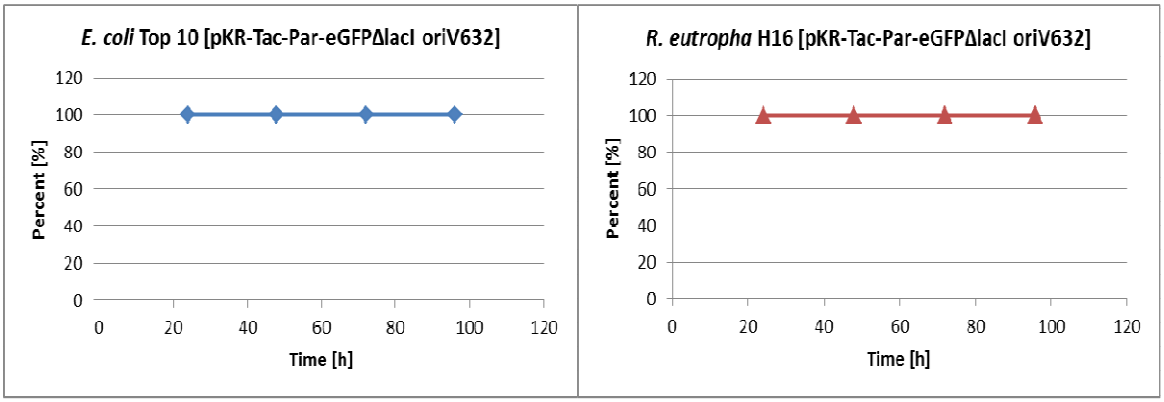


Figure 35: Stability of the plasmid pKR-Tac-Par-egfpΔlacI oriV632 in E.coli Top10 and R. eutropha H16 over the time period of 96 h.

The results of plasmid stability assay for the plasmid pKR-Tac-Par-eGFPΔlacI RSF1010 in *E. coli* Top 10 and *R. eutropha* H16 are shown in *Table 20* and *Table 22*.

Table 20: Results of the plasmid stability assay of the E. coli Top 10 [pKR-Tac-Par-egfpΔlacI RSF1010] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	3.74 [a]	3.84	3.82	3.77	3.89
Generation time [h]	-	5.6	5.6	5.7	5.6
number of generations in 24 h	-	4.3	4.3	4.2	4.3
CFUs on LB (1)	$3*10^7$	$19*10^6$	$8*10^6$	$15*10^6$	$17*10^6$
CFUs on LB + Kan 40µg/mL (1)	$3*10^7$	$19*10^6$	$8*10^6$	$15*10^6$	$17*10^6$
CFUs on LB (2)	$13*10^6$	$26*10^6$	$17*10^6$	$13*10^6$	$15*10^6$
CFUs on LB + Kan 40µg/mL (2)	$13*10^6$	$26*10^6$	$17*10^6$	$13*10^6$	$15*10^6$
CFUs on LB (3)	$3*10^7$	$18*10^6$	$19*10^6$	$12*10^6$	$16*10^6$
CFUs on LB + Kan 40µg/mL (3)	$3*10^7$	$18*10^6$	$19*10^6$	$12*10^6$	$16*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**5.6**		
Average no. of generations in 24 h			**4.3**		
Average no. of generations in 96 h			**17**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Table 21: Results of the plasmid stability assay of the R. eutropha H16 [pKR-Tac-Par-egfpΔlacI RSF1010] strain and its average generation time as well as the number of generations in 24 h and 96 h.

	0 h	24 h	48 h	72 h	96 h
MW OD_{600} (precultures)	2.23 [a]	4.65	5.02	5.2	5.1
Generation time [h]	-	5.3	5.2	5.2	5.1
number of generations in 24 h	-	4.5	4.6	4.6	4.7
CFUs on TSB + Gen 20 µg/mL (1)	$4*10^6$	$14*10^6$	$8*10^6$	$11*10^6$	$17*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (1)	$4*10^6$	$14*10^6$	$8*10^6$	$11*10^6$	$17*10^6$
CFUs on TSB + Gen 20 µg/mL (2)	$12*10^6$	$12*10^6$	$5*10^6$	$8*10^6$	$9*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (2)	$12*10^6$	$12*10^6$	$5*10^6$	$8*10^6$	$9*10^6$
CFUs on TSB + Gen 20 µg/mL (3)	$12*10^6$	$11*10^6$	$11*10^6$	$5*10^6$	$9*10^6$
CFUs on TSB + Gen 20 µg/mL + Kan 200 µg/mL (3)	$12*10^6$	$11*10^6$	$11*10^6$	$5*10^6$	$9*10^6$
Plasmid stability in 96 h [%]			**100**		
Average Generation time [h]			**5.2**		
Average no. of generations in 24 h			**4.6**		
Average no. of generations in 96 h			**18**		

[a] Here the OD_{600} of the preculture is given, which was inoculated with a single colony.
(1)-(3) mark the results of the triple determination.

Both strains *E. coli* Top10 and *R. eutropha* H16, containing the pKR-Tac-Par-eGFPΔlacI RSF1010 vector, showed 100 % stability during the plasmid stability assay (see *Figure 36*). The average generation time for *E.coli* Top10 [pKR-Tac-Par-eGFPΔlacI RSF1010] and *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] was determined as 6.4 h and 5.75 h, respectively. This led to an average generation number of 15 generations in the *E.coli* Top10 strain and 17 generations in the *R. eutropha* H16 strain during 96 h.

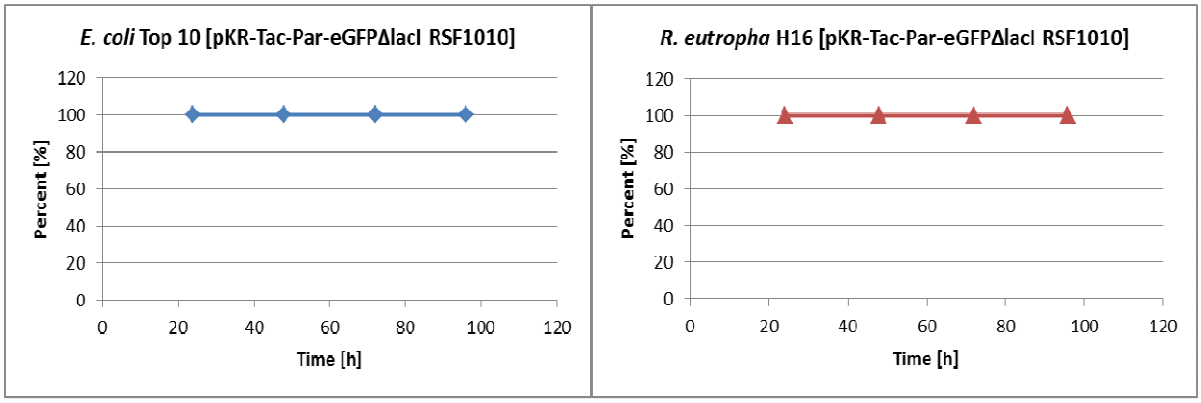


Figure 36: Stability of the plasmid pKR-Tac-Par-egfpΔlacI RSF1010 in E.coli Top10 and R. eutropha H16 over the time period of 96 h.

In the following *Table 22* the results of the average generation time per hour as well as the number of generations in 96 h for all four expression vectors in *E. coli* Top10 or *R. eutropha* H16 are listed.

Table 22: Results of the average generation time per h and the number of generations in 96 h for all four expression vectors.

Vector	*E. coli* Top10		*R. eutropha* H16	
	Average generation time [h]	Generations in 96 h	Average generation time [h]	Generations in 96 h
pKR-Tac-Par-eGFPΔlacI REP	6.4	15	5.9	16
pKR-Tac-Par-eGFPΔlacI oriV393	6.3	16	4.5	18
pKR-Tac-Par-eGFPΔlacI oriV632	6.4	15	5.75	17
pKR-Tac-Par-eGFPΔlacI RSF1010	6.4	15	5.75	17

Regarding the results for the different *E. coli* Top 10 strains there is almost no difference in the generation time and the number of generations which are passed during the 96 h of the plasmid stability test. All *E. coli* Top10 strains, containing the expression vectors with the different origins of replication have an average generation time of 6.3 - 6.4 h which equate

to 15-16 generations in 96 h. Here the different origins or replication seem to have no effect on the growth rate of the *E. coli* Top10 strains.

For the *R. eutropha* H16 strains a slight difference between *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] and the strains with the other three origins could be detected. The *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] strain has the shortest generation time with only 4.5 h and passes 18 generations during the plasmid stability test duration. The other strains *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP], *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI oriV632] and *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] are growing slightly slower and have a longer generation time of 5.75 – 5.9 which is 1.3 times higher than for the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] strain.

3.3 Detection of eGFP

3.3.1 Colony PCR of *egfp*

For the analysis of the *egfp* gene in the *R. eutropha* H16 strains with the different expression vectors liquid cultures or single colonies were used as samples for the colony PCR. The sample preparation, the different reaction components and the cPCR programm is described in *Table 8 and Figure 6*. On the following agarose gel the results of the colony PCR for *egfp* gene (size: ~600 bp) of the strains *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] (lane 3), *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] (lane 4) and *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV632] (lane 5) are shown. As positive control the *E.coli* Top 10 [pKR-Tac-Par-eGFPΔlacI REP] strain (lane 1) and as negative control the empty *R. eutropha* H16 wild type strain was used (lane 2).

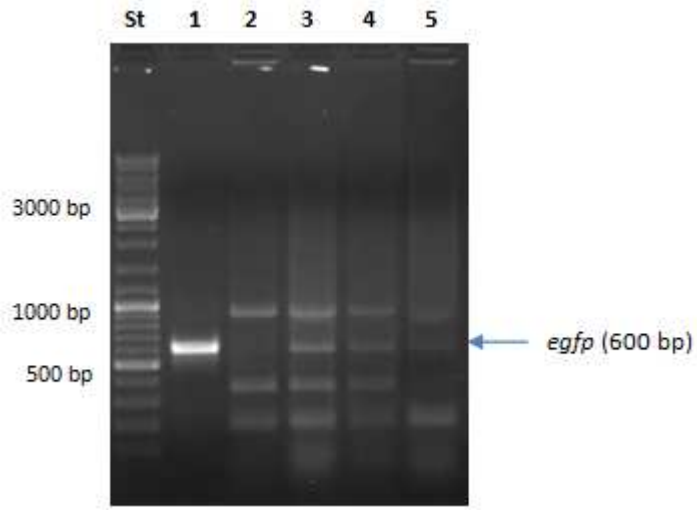


Figure 37: Agarose gel of colony PCR for egfp: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: E. coli Top 10 [pKR-Tac-Par-EGFPΔlacI Rep] positive control; lane 2: R. eutropha H16 wild type strain, negative control; lane 3: R. eutropha H16 [oKR-Tac-Par-egfpΔlacI oriV393]; lane 4: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI Rep], lane 5: R. eutropha H16 [oKR-Tac-Par-egfpΔlacI oriV632]

Regarding the agarose gel the colony PCR of the *E.coli* Top10 [pKR-Tac-Par-eGFPΔlacI REP] strain (lane1) resulted in a clear *egfp* band at ~600 bp. For the *R. eutropha* H16 strains additional unspecific bands at 200, 400 and 1000 bp are visible on the gel. However, the *egfp* band was clearly visible for all *R. eutropha* H16 strains, containing the three different vectors pKR-Tac-Par-eGFPΔlacI oriV393 (lane 3), pKR-Tac-Par-eGFPΔlacI REP (lane 4) and pKR-Tac-Par-eGFPΔlacI oriV632 (lane5) whereas in the negative control (lane 2) only the additional

bands at 200, 400 and 1000 bp occurred. Despite the variation of the PCR conditions regarding the annealing temperature and extension time or the application of other primer combinations, the additional bands always occurred. For the *R. eutropha* H16 strain containing the pKR-Tac-Par-eGFPΔlacI RSF1010 vector the performed colony PCRs showed no *egfp* band.

However, for the *R. eutropha* H16 strain, where the pKR-Tac-Par-eGFPΔlacI RSF1010 vector was introduced via conjugation an intense *egfp* band could be detected via the performed colony PCR (see *Figure 38*). As positive control the *E.coli* Top 10 [pKR-Tac-Par-eGFPΔlacI RSF1010] strain (lane 1) was used. In lane 2-6 five different clones of *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] from an agar plate were used as template for the PCR.

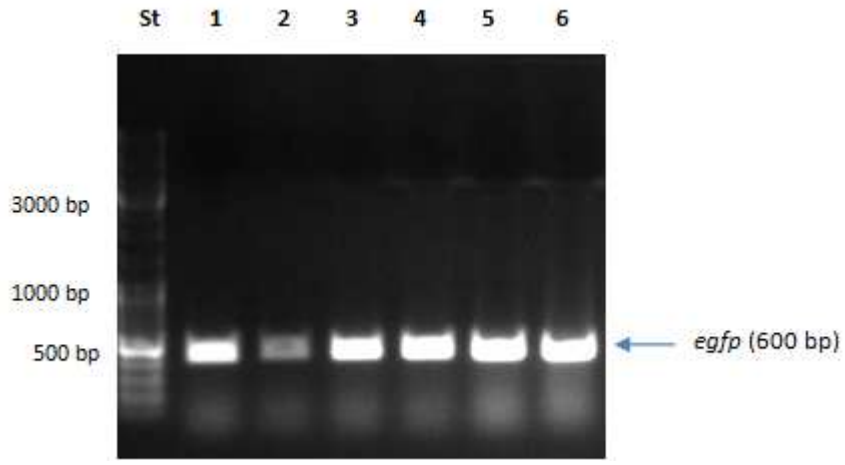


Figure 38: Agarose gel of colony PCR for egfp: St. GeneRuler™ DNA Ladder Mix, lane 1: E. coli Top 10 [pKR-Tac-Par-EGFPΔlacI Rep] positive control; lane 2-6: R. eutropha H16 [oKR-Tac-Par-egfpΔlacI RSF1010]

At the agarose gel for all five *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] clones the *egfp* band at 600 bp was clearly visible. Furthermore no additional bands, like in the former colony PCRs for the other *R. eutropha* H16 strains were visible, although the same PCR conditions were applied.

3.3.2 Fluorescence microscopy for eGFP

In order to detect eGFP expression in the *R. eutropha* H16 strains with the new designed expression vectors the Leica DM LB2 microscope (Wetzlar, Germany) with the HCX FL Fluotar 100x/1.30 oil objective in phase contrast (PH3) mode was used. EGFP was excited via a high pressure mercury lamp using emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue) with an exposure time of 290 ms. For the image

collection the Leica DFC350 FX monochrome Digital Camera was employed. All shown images were amplified eightfold. The *R. eutropha* H16 strains containing the expression vectors with the four different origins of replication were grown in liquid cultures till an OD_{600} of 0.4 to 2.5.

In *Figure 39* the visible light (A) and the fluorescence light (B) images of the wild type *R. eutropha* H16 strain are shown as negative control.

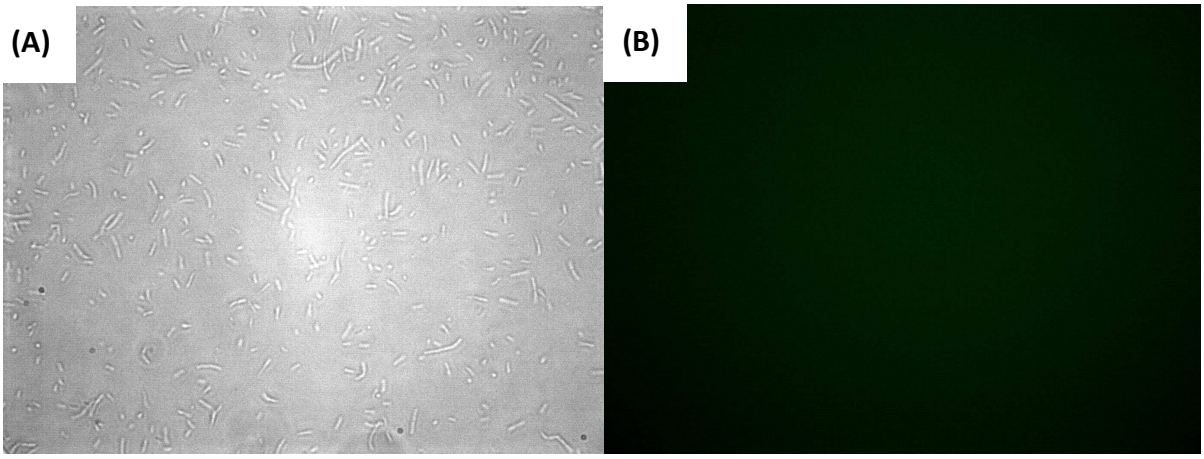


Figure 39: Fluorescence microscopy of R. eutropha H16 wildtype: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms.The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

In the visible light image the *R. eutropha* H16 cells in various length and different growth stages are visible. Regarding the fluorescence light image clearly no fluorescing cells are visible.

In *Figure 40* the fluorescence microscopy images of the *R. eutropha* H16 strain containing the pKR-Tac-Par-eGFPΔlacI REP vector are shown.

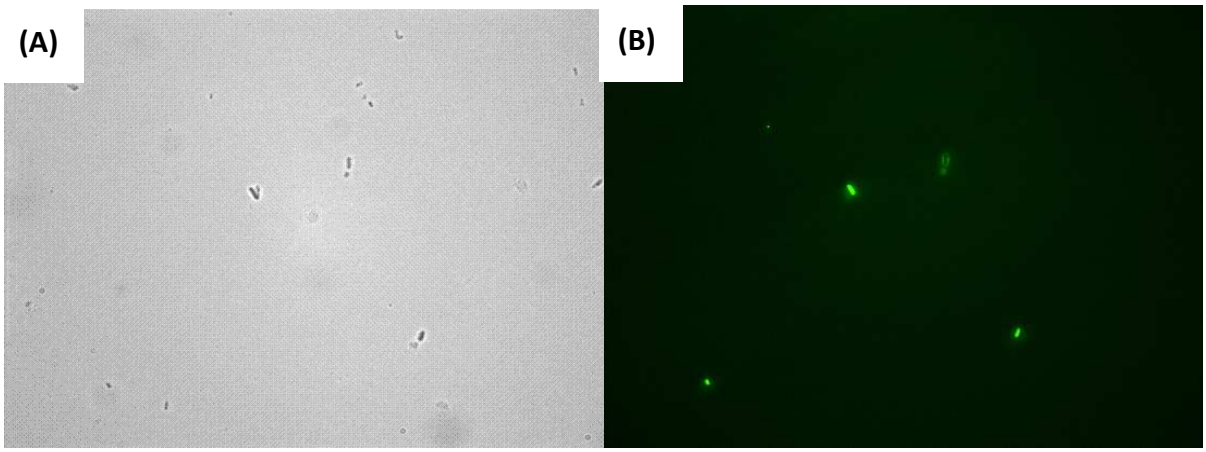


Figure 40: Fluorescence microscopy of R. eutropha H16 [pKR-Tac-Par-egfpΔlacI REP]: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms. The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

In the first picture (A) a few *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP REP] cells are visible. The OD_{600} of 0.35 was very low, however, looking at the corresponding fluorescence image (B) the cells showed an intense fluorescence signal. Despite the fact that eGFP was successfully expressed in the cells it has to be considered that not all visible cells in image A were fluorescing in image B.

The resulting images of the fluorescence microscopy of the *R. eutropha* H16 strain containing the pKR-Tac-Par-eGFPΔlacI oriV393 vector are shown in *Figure 41*.

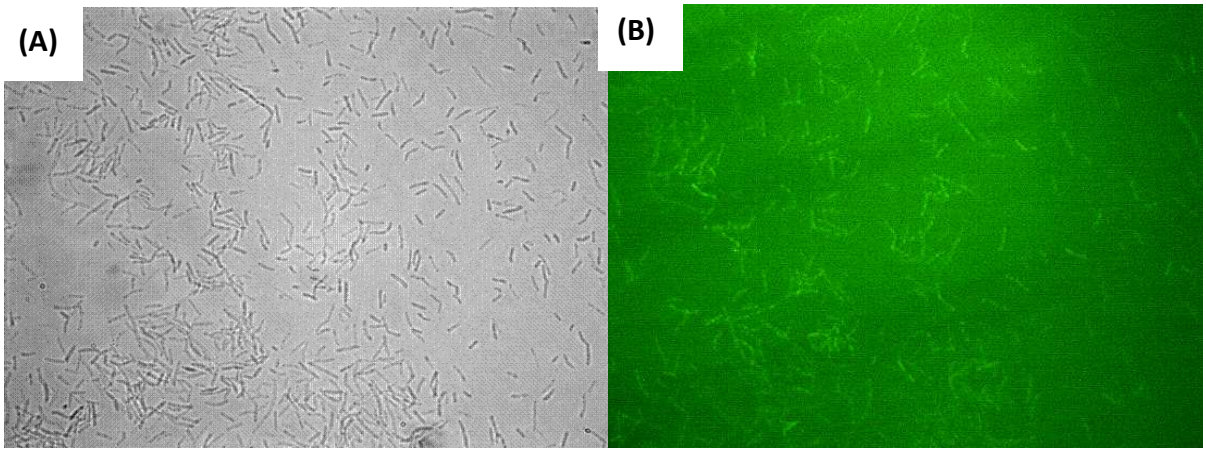


Figure 41: Fluorescence microscopy of R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV393]: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms. The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

In the visible light image (A) many *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP oriV393] cells are visible. The liquid culture had an OD_{600} of about 1.2. Regarding the corresponding fluo-

rescence image (B) the cells were fluorescing and eGFP was expressed. However not all cells, visible in the transmitted light showed a signal in the fluorescing light. Furthermore the fluorescence was not as intense as in the picture of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP REP] cells before.

In *Figure 41* the visible light (A) and the fluorescence light (B) images of the *R. eutropha* H16 strain containing the pKR-Tac-Par-eGFPΔlacI oriV632 vector are shown.

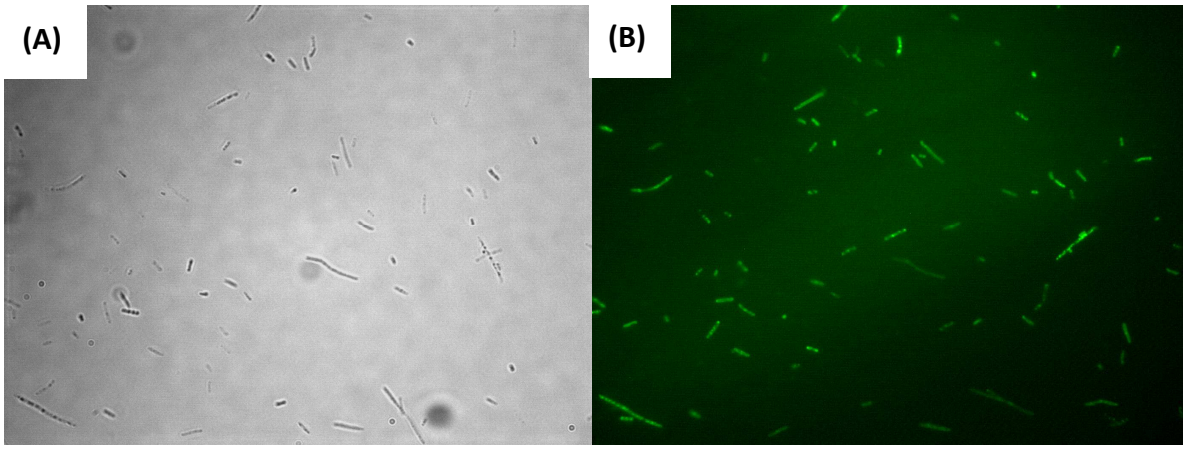


Figure 42: Fluorescence microscopy of R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV632]: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms. The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

In the first picture (A) *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP oriV632] cells in different lengths are visible. The OD_{600} of the liquid culture was with 0.9 rather low. Nevertheless, looking at the corresponding fluorescence image (B) the cells showed an intense fluorescence signal. Through comparison of the two corresponding images the cells still seemed to be very agile because they slightly changed their position between the collection of the visible light image and the fluorescence light image. Moreover almost all visible cells showed also a fluorescence signal.

The microscopy images of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] with visible light (A) and fluorescence light (B) are shown in *Figure 43*.

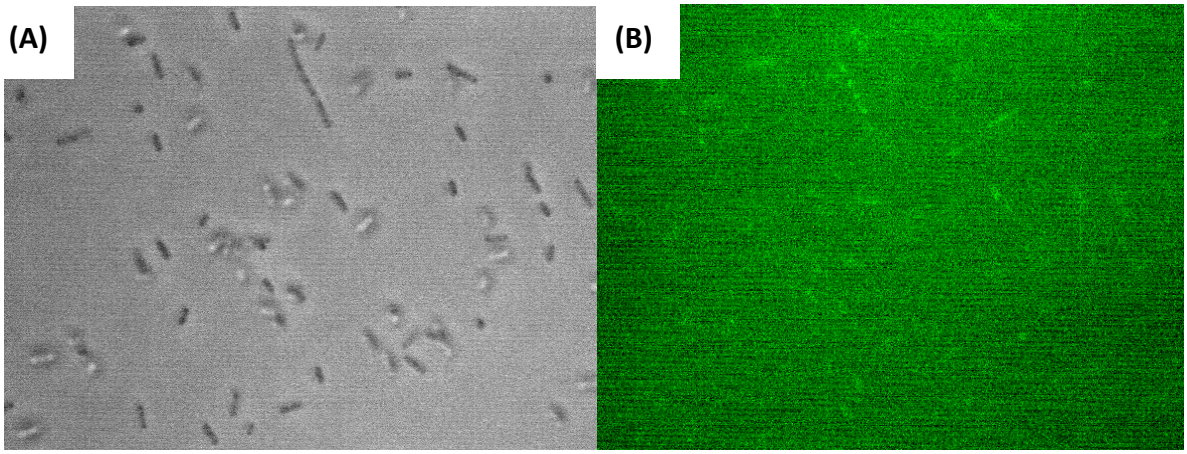


Figure 43: Fluorescence microscopy of R. eutropha H16 [pKR-Tac-Par-egfpΔlacI RSF1010]: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms. The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

In the visible light image (A) many *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP RSF1010] cells varying in their length are visible. The liquid culture had an OD_{600} about 0.5. Regarding the corresponding fluorescence image (B) the cells were just slightly fluorescing. The fluorescence signal was rather weak compared to the signal of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP REP] or *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP oriV632] cells before. Furthermore not all cells, visible in the transmitted light showed a signal in the fluorescing light.

However, the pKR-Tac-Par- eGFPΔeGFP RSF1010 vector was also introduced into *R. eutropha* H16 via conjugation using the *E.coli* S17-1 as donor strain and the microscopy images of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] generated via conjugation are shown in *Figure 44*.

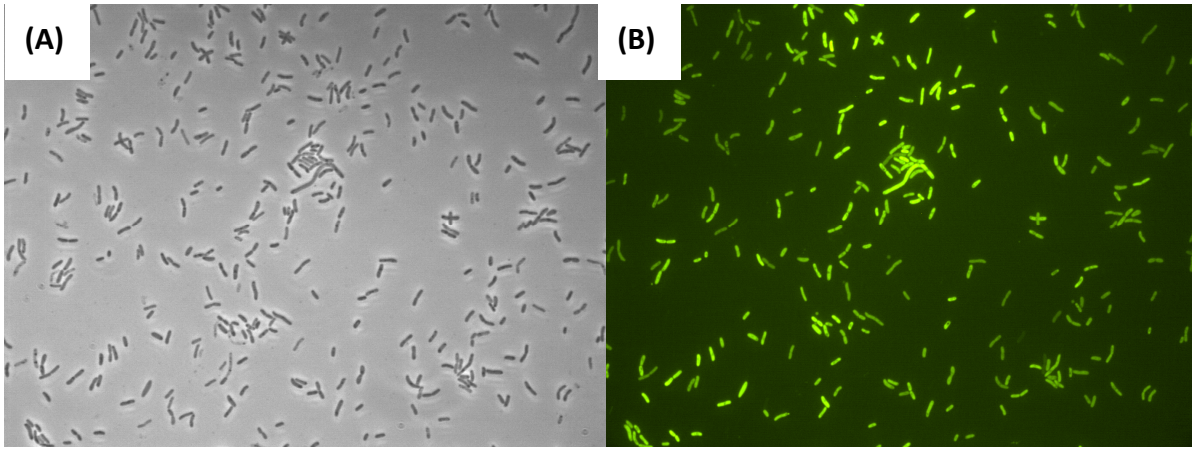


Figure 44: Fluorescence microscopy of R. eutropha H16 [pKR-Tac-Par-egfpΔlacI RSF1010] generated via conjugation: (A) Visible light, (B) emission bandpass filter at 450/490 nm and suppression longpass filter at 515 nm (Filter I3, blue), exposure time 290ms. The images were amplified eightfold using the Leica DFC350 FX monochrome Digital Camera and the Corel photo editor software.

For the microscoping of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP RSF1010] strain generated via conjugation the cells were directly taken from a single colony. In the visible light image (A) many *R. eutropha* H16 [pKR-Tac-Par-eGFPΔeGFP RSF1010] cells were visible. Regarding the fluorescing light image (B) all cells showed a very intense fluorescing signal which indicated the presence of the conjugated vector and a strong eGFP expression.

Finally it has to be mentioned that the shown images of the different strains were collected at different dates and the settings of the microscope were often modified by its different users. Despite a short justification of the microscope before each measurement the settings at the collection of the different images could have been varied slightly, which has to be considered for the evaluation of the fluorescence intensity.

3.3.3 Results of SDS-PAGE and Western Blot analysis

Another method for the detection of eGFP in the *R. eutropha* H16 strains was the SDS-PAGE and the Western Blot analysis. In *Figure 45* the SDS-PAGE gel (A) and the corresponding Western Blot (B) for the following strains: *R. eutropha* H16 [pKR-Lac-Par-eGFPΔlacI REP] (lane 3), *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] (lane 4 and 5), *R. eutropha* H16 [pKR-CIV1a-Par-eGFPΔlacI REP] (lane 6), *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] (lane7), *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV632] (lane 8 and 9) are shown. As positive control the *E. coli* Top 10 [pKR-Tac-Par-eGFPΔlacI REP] (lane 1) and as negative control the emp-

ty *R. eutropha* H16 wild type strain (lane 2) were employed. Beside the *R. eutropha* H16 strains with the new designed vectors from this work also strains containing the pKR-Lac-Par-eGFPΔlacI REP and the pKR-CIV1a-Par-eGFPΔlacI REP vector were loaded on the gel. In the first vector the Tac promoter was replaced with the common Lac promoter, whereas the second vector contained the CIV1a promoter originating from *R. eutropha* H16 (derived by Steffen Gruber). The vector maps off the additional expression vectors are shown in *Appendix 2: Vector maps*).

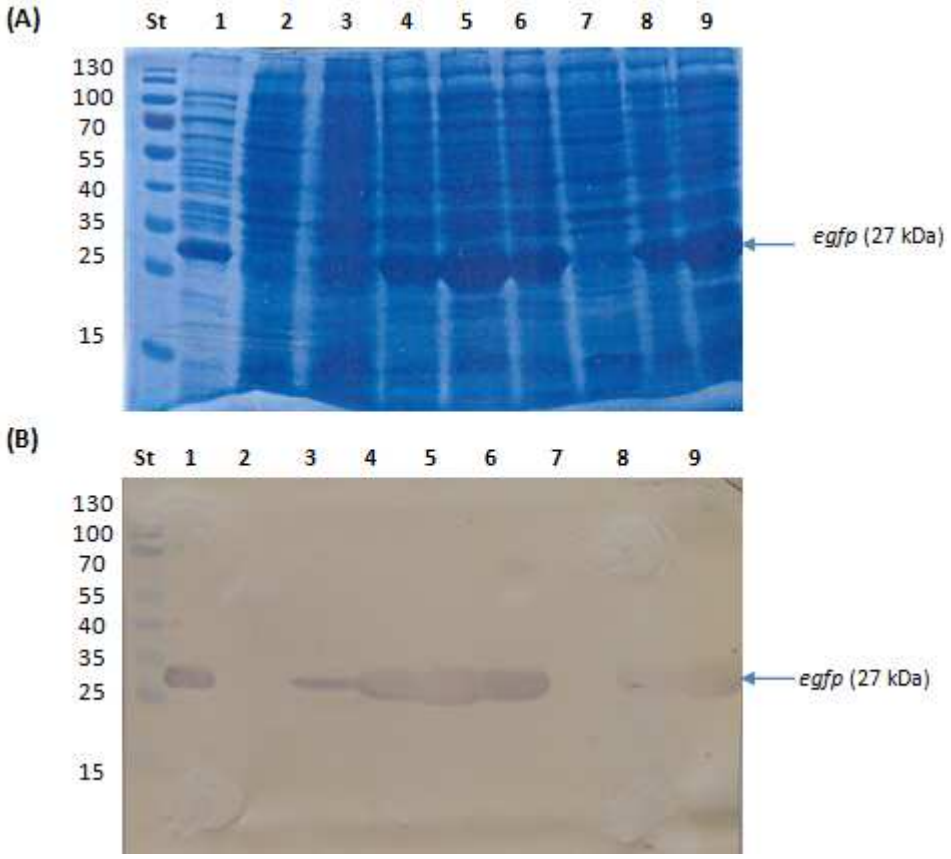


*Figure 45: SDS-PAGE gel **(A)** and Western Blot **(B)** for the detection of egfp in various R. eutropha H16 strains. The sample order is similar on both pictures: St. PageRuler Prestained Protein Ladder (Fermentas), lane 1: E.coli Top10 [pKR-Tac-Par-egfpΔlacI REP] (positive control), lane 2: R. eutropha H16 (negative control), lane 3: R. eutropha H16 [pKR-Lac-Par-egfpΔlacI REP], lane 4: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI Rep], lane 5: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI REP], lane 6: R. eutropha H16 [pKR-CIV1a-Par-egfpΔlacI Rep], lane 7: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV 393], lane 8: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV632], lane 9: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI oriV632]*

In the SDS-PAGE as well as in the Western Blot the eGFP band (at 27 kDa) is clearly visible for all *R. eutropha* H16 strains containing the new designed expression vectors. The most intense eGFP band is visible in lane 5 corresponding to the strain *R. eutropha* H16 [pKR-Tac-

Par-eGFPΔlacI REP]. In lane 6 the *R. eutropha* H16 [pKR-CIV1a-Par-eGFPΔlacI REP] strain showed also a high expression level of eGFP. This vector contained the homologous CIV1a promoter from *R. eutropha* H16. The eGFP expression of the strains containing the origins of replication form the RK2 plasmid oriV393 and oriV632 are shown in lane 7 and lane 8+9. For the smaller form of the RK2 origin oriV393 just a very slight band on the SDS-PAGE gel and almost no visible band on the Western Blot could be detected. However, both samples of the *R. eutropha* H16 strain containing the vector with the regular RK2 origin oriV632 (lane 8 and 9) exhibited an intense eGFP band on the SDS-PAGE gel and the Western Blot.

For the strain *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] (vector introduced via electro transformation) no positive signal for eGFP neither on the SDS-PAGE nor on the Western Blot results could be achieved. However, this vector was also introduced in the *R. eutropha* H16 cells via conjugation and the resulting *R. eutropha* H16 strain [pKR-Tac-Par-eGFPΔlacI RSF1010] showed a very intense eGFP band on the SDS-PAGE gel as well as on the Western Blot. In the first approach the *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] cell pellets were prepared like mentioned in 2.6.2 – the cell pellet was resuspended in the 15-fold amount of potassium phosphate buffer – but the resulting SDS-PAGE gel was quite overloaded (Data not shown). Therefore the samples were prepared like the *E. coli* [pKR-Tac-Par-eGFPΔlacI RSF1010] positive control - the cell pellet was resuspended in the 33.3-fold amount of potassium phosphate buffer – without any up-concentration. And the same amounts of resuspended cells (3 µL) were loaded onto the gel. In *Figure 46* the SDS-PAGE gel and the Western Blot of seven different clones (lanes 3-9) of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] strain are shown. The *E. coli* [pKR-Tac-Par-eGFPΔlacI RSF1010] positive control was loaded in lane 1 and the empty *R. eutropha* H16 wild type strain (lane2) was used as negative control.

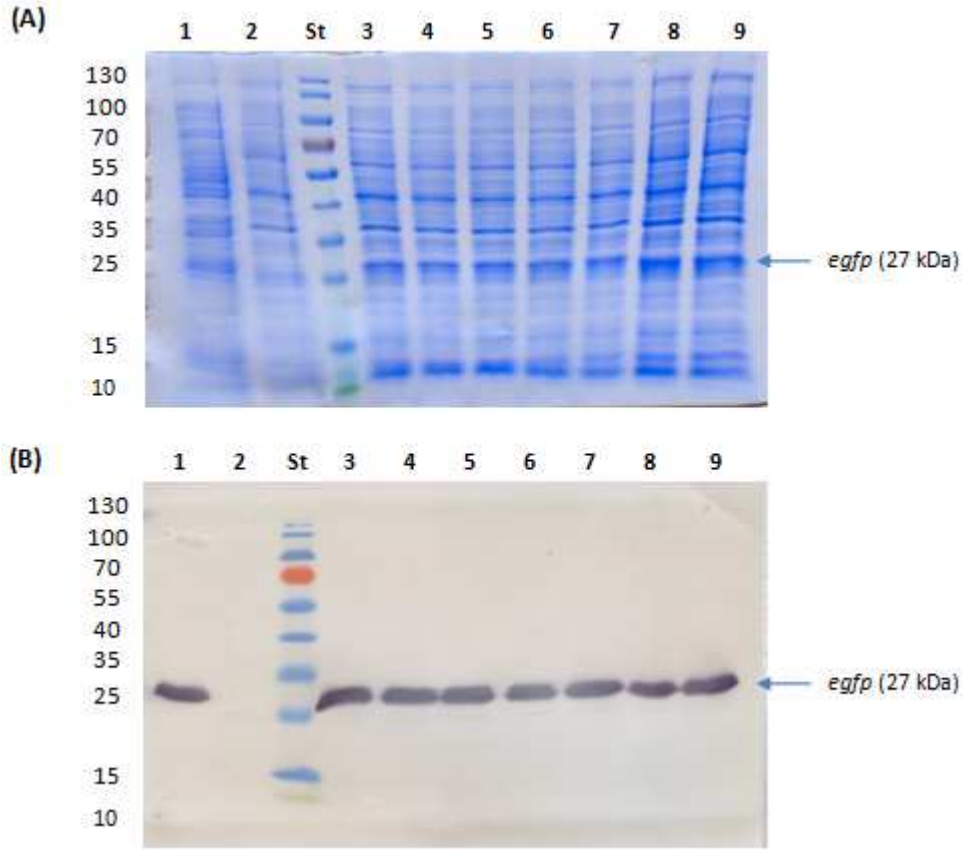


*Figure 46: SDS-PAGE gel **(A)** and Western Blot **(B)** for the detection of egfp in different R. eutropha H16 strains. The sample order is similar on both pictures: St. PageRuler Prestained Protein Ladder (Fermentas), lane 1: E.coli Top10 [pKR-Tac-Par-egfpΔlacI REP] (positive control), lane 2: R. eutropha H16 (negative control), lanes 3-9: R. eutropha H16 [pKR-Tac-Par-egfpΔlacI RSF1010].*

Regarding the SDS-PAGE as well as the Western Blot a very intensive eGFP band (at 27 kDa) is clearly visible for all *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] clones. Considering the sample preparation the same amounts of cells were applied for the *R. eutropha* H16 samples as well as for the *E. coli* positive control. Therefore a rough comparison of the different eGFP expression levels was possible. The produced amount of eGFP in the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] clones seemed to be similar to the amounts of the *E. coli* positive control.

3.3.4 Detection of the Fluorescing Units

The *R. eutropha* H16 strains containing the designed expression vectors were cultivated to an OD of 1.0 – 3.5 in order to compare their fluorescence. After cell disruption the fluorescing units (FU) of the cell lysate were measured as described in 2.6.4. For the determina-

tion of the relative fluorescing units (RFU) the obtained FUs from the negative control were subtracted from the measured FUs from the particular samples.

In the first approaches the cultures were grown in liquid LB media. The resulting FUs for the *R. eutropha* H16 strains containing expression vectors with different origins of replications (*R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP], *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV393], *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV632]) and with different promoters (*R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP], *R. e.* H16 [pKR-Lac-Par-eGFPΔlacI REP], *R. e.* H16 [pKR-ClV1a-Par-eGFPΔlacI REP]) are shown in *Table 23.* As negative controls the *R. eutropha* H16 wild type strain as well as the *R. eutropha* H16 [pKR-ClV1a-Par-ΔeGFPΔlacI REP] strain with the deleted *egfp* were employed.

Table 23: Results of the Fluorescing Units (FU) for different R. eutropha H16 strains grown in LB media.

Strains	**OD_{600}**	**FU**	**RFU[a]**
***R. e.* H16 wild type**	0.7	253	-
	1.2	309	-
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP]**	1.1	413	+104
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV393]**	1.2	599	+290
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV632]**	1.0	374	+65
***R. e.* H16 [pKR-Lac-Par-eGFPΔlacI REP]**	0.8	353	+100
***R. e.* H16 [pKR-ClV1a-Par-eGFPΔlacI REP]**	1.0	322	+13
***R. e.* H16 [pKR-ClV1a-Par-ΔeGFPΔlacI REP]**	1,2	298	-

[a] *The RFU were calculated by subtracting the FU of the negative control with the best matching OD_{600} (253 at OD_{600} 0.7 or 309 at OD_{600} 1.2) from the measured FUs of the different strains.*

Despite the normalization of the OD_{600} before the measurement the resulting FUs of *R. eutropha* H16 strains grown in LB media seemed to depend on the particular OD_{600}. For the negative control *R. eutropha* H16 the FU varied from 253 at an OD_{600} of 0.7 to an FU of 309 at an OD_{600} of 1.2. Although the samples originated from the same flask culture the difference of 0.5 OD_{600}-units resulted in an increase of the FUs of about 50 units. The second negative control, *R. eutropha* [pKR-Tac-Par-ΔeGFPΔlacI REP], had an average FU of 298 at a primary OD_{600} of 1.2. In order to calculate the relative fluorescing units the FU of the negative control with the best matching OD_{600} (253 at OD_{600} 0.7 or 309 at OD_{600} 1.2) was subtracted from the measured FUs of the different strains. The highest relative units of 290 were obtained with the *R. eutropha* [pKR-Tac-Par-eGFPΔlacI orV393]. *R. eutropha* [pKR-Tac-Par-

eGFPΔlacI REP] and *R. eutropha* [pKR-Lac-Par-eGFPΔlacI REP] showed also quite high RFUs with 104 and 100, respectively. In the two remaining strains *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV632] and *R. e.* H16 [pKR-CIV1a-Par-eGFPΔlacI REP] the gained RFU values of +65 and +13 were rather low compared to the deviation range of the negative control of 50 FU. Therefore it is not clear if the measured FUs were a signal of eGFP expression or occurred due to measurement errors and the high background of the LB media.

Due to the dependence of the FUs of the negative controls on the OD_{600} and the high background values the *R. eutropha* strains were cultivated in MSM media for the second approach. The resulting FUs from the negative controls (*R. e.* H16 wild type and *R. e.* H16 [pKR-CIV1a-Par-ΔeGFPΔlacI REP]) as well as the FUs and RFUs from the strains containing the different expression vectors are listed in *Table 24*.

Table 24: Results of the Fluorescing Units (FU) for different R. eutropha H16 strains grown in MSM.

Strains	OD_{600}	FU	RFU[a]
***R. e.* H16 wild type**	1.1	180	-
	2.0	204	-
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP]**	1.2	299	+107
	1.6	328	+136
	4.2	317	+125
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV393]**	1.2	293	+101
***R. e.* H16 [pKR-Tac-Par-eGFPΔlacI orV632]**	1.0	260	+68
***R. e.* H16 [pKR-Lac-Par-eGFPΔlacI REP]**	1.0	276	+84
***R. e.* H16 [pKR-CIV1a-Par-eGFPΔlacI REP]**	1.3	251	+59
***R. e.* H16 [pKR-CIV1a-Par-ΔeGFPΔlacI REP]**	1,8	206	-
	3.5	194	-

[a] *The RFU were calculated by subtracting the average FU of the R. e. H16 wild type strain (negative control) of 192 from the measured FUs of the different strains.*

Regarding the results of the empty *R. eutropha* H16 strain the FUs seem independent of the OD_{600}. For the negative control 180 FU were measured at an OD_{600} of 1.1 and 204 FU at an OD_{600} of 2.0, respectively. Considering some unavoidable deviations during the cell disruption and the measurement the difference of 20 FU could be neglected. For the calculation of the RFUs the average value of 192 from the FUs of the negative control was used. This value matched perfectly to the measured FUs of the second negative control *R. e.* H16 [pKR-CIV1a-

Par-ΔeGFPΔlacI REP] of 194 at an OD_{600} of 1.6 and of 206 at an OD_{600} of 3.5. The highest RFUs were reached by the *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI REP] strain which was measured at three different ODs_{600}: +107 at an OD_{600} of 1.2, +136 at an OD_{600} of 1.6 and +125 at an OD_{600} of 4.2. With a RFU of +101 the *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI oriV393] strain showed also a strong fluorescence signal. The other strains *R. e.* H16 [pKR-Tac-Par-eGFPΔlacI oriV632], *R. e.* H16 [pKR-Lac-Par-eGFPΔlacI REP] and *R. e.* H16 [pKR-CIV1a-Par-eGFPΔlacI REP] reached RFU values of +68, +84 and +59, which were still higher than the deviation between the measured FUs of the two negative controls. Therefore the expression of eGFP could be successfully detected in all tested *R. eutropha* H16 strains.

3.4 Detection of LacZ

Due to some problems with the detection of *egfp* – weak fluorescence signals during microscopy and high background signals at the measurement of the fluorescing units – *lacZ* was introduced into one expression vector as alternative reporter gene. The pKR-Tac-Par-LacZΔlacI REP vector was successfully cloned into *E.coli* Top10 and the detection of positive transformants was managed via plating out on X-gal LB agar plates. Via the enzymatic conversion of the X-gal into a blue indigo dye by the β-galactosidase the best clones, showing a high expression level of LacZ (dark blue color) were selected and transformed into *R. eutropha* H16.

For the detection of LacZ in the *R. eutropha* H16 [pKR-Tac-Par-LacZΔlacI REP] different transformants were cultivated in LB media and the cells were loaded on a SDS-PAGE gel. As positive control *E. coli* Top10 cells containing the pKR-Tac-Par-LacZΔlacI REP vector were applied. Several SDS-PAGE gels with different cultures of *R. eutropha* H16 [pKR-Tac-Par-LacZΔlacI REP] were performed, however, no LacZ band could be detected. (Data not shown)

As second detection method the β-galactosidase assay was conducted. Hereby the *R. eutropha* H16 [pKR-Tac-Par-LacZΔlacI REP] transformants were cultivated in minimal medium and prepared like already mentioned in *2.7*. As positive control *E. coli* Top10 cells containing the pKR-Tac-Par-LacZΔlacI REP vector were applied. Several transformants were tested for

the presence of β-galactosidase but no yellow color change indicating the enzymatic conversion of oNPG to oNP was observed. Therefore the expression of *lacZ* could not be detected.

As last approach the *lacZ* gene was detected via PCR. The transformants of *R. eutropha* H16 [pKR-Tac-Par-LacZΔlacI REP] were streaked out for plasmid isolation and the isolated plasmid DNA was used as template. In *Table 5* the PCR conditions are listed and in *Figure 47* the agarose gel of the PCR product is shown.

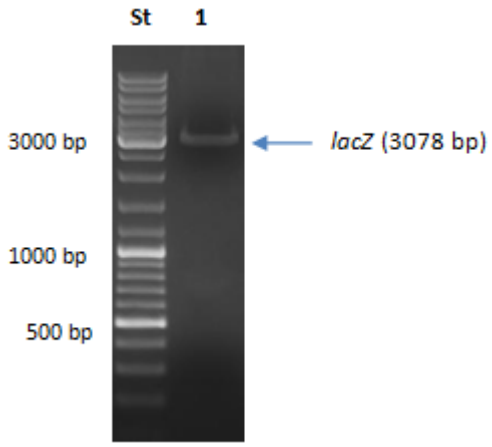


Figure 47: Agarose gel of the PCR for the lacZ gene on the pKR-Tac-Par-lacZΔacI REP vector isolated from R. eutropha H16 [pKR-Tac-Par-lacZΔacI REP]: St. GeneRuler™ DNA Ladder Mix 500 ng, lane 1: lacZ (3078 bp)

In lane 1 the band of the *lacZ* gene (at 3078 bp) was clearly visible. This was a proof for the successful transformation of the pKR-Tac-Par-LacZΔlacI REP vector into *R. eutropha* H16.

4 Discussion

The gram-negative and respiratory facultative lithoautotrophic soil and freshwater bacterium *Ralstonia eutropha* H16 has attracted significant interest because of its versatility regarding different growth modes. Due to its ability to grow to high cell densities during litoautotrophic or heterotrophic conditions *R. eutropha* H16 can be used as host for heterologous gene expression and production organism [15]. Several attempts applying constitutive expression systems, based on broad-host-range plasmids and using the Tac or Lac promoter, for the expression of heterologous genes in *R. eutropha* H16 were already performed [16–18]. Due to the lack of transport capacities for inducers like lactose or IPTG expression systems are not inducible [8], [9]. Other expression system employing different promoters like the P_{phaC} or P_{phaP} derived from *R. eutropha* H16 were also reported [18–21].

However, due to the fact that the complex inducible systems prohibited their application as simple expression system under different growth conditions the research group of Petra Köfinger started with the construction of simple expression vectors for *R. eutropha* H16 using the P_{Tac} or the P_{Lac} as constitutive promoter. The vectors contained the kanamycin resistance gene as selective antibiotic marker and *egfp* as reporter gene for monitoring the expression levels. The main focus of this work was the cloning of four different origins of replication originating from various broad-host-range plasmids generating new expression vectors, as well as the comparison and the analysis of their replication and stable maintenance in *R. eutropha* H16. For an additional increase of the plasmid stability the partitioning sequence *par* from the RK2 plasmid was also cloned into the vectors. In order to monitor and compare the effect of different replication systems on the expression levels of the new vectors the amounts of expressed eGFP were detected via different detection methods like SDS-PAGE, Western Blot analysis and the measurement of the fluorescing units.

Construction and expression of the new designed vectors in R. eutropha H16

The following different origins of replication were chosen for the expression vectors: the origin of the replication REP of the medium copy number plasmid pBBR1, the low-copy origins of the RK2 plasmid (IncP family) – oriV393 and oriV632 – (shown in *Figure 2*) and the rather high-copy number mini-replicon of the RSF1010 plasmid (IncQ family) – called RSF1010. All used origins of replication were originating from broad-host-range plasmids and were already proved to replicate in *R. eutropha* H16 [27–29], [46], [51], [52], except for the small variant of the mini-replicon from the RK2 plasmid oriV393, which was just applied in *E. coli* and *Pseudomonas aeruginosa* [35].

After sequencing of the two origins of replication from the RK2 plasmid – oriV393 and oriV632 – differences between our sequence and the original sequence of the RK2 plasmid [30] were detected. The origins were amplified from the RK2 plasmid from the IMBT culture collection (#381) via an overlap extension PCR in order to remove the tetracycline resistance gene located between the *oriV* sequence and the trfA operon. Therefore, the mutations may have been introduced via PCR. However, several PCR products of the oriV393 and oriV632 were sequenced showing all the same mutations: a base exchange from adenosine to cytosine at position 180/347 (oriV393/oriV632) located between two iterons sequences and an insertion of an additional tyrosine base at position 347/585 (oriV393/oriV632) in the GC-rich region. In the oriV632 an additional mutation occurred in the part of the sequence, which is missing in the oriV393. The base exchange from adenosine to tyrosine (at position 138) was located 20 bp upstream of the first iteron sequence. The sequence of oriV393 and oriV632 with the marked mutations shown in *Appendix 3: Sequences*.

Mutations in the origin of replication could have different effects on the replication system, like de- or increase of the copy number or even the total abolishment of the replication activity. For the origins of the RK2 plasmid a lot of research was performed in the past years regarding mutations in the sequence of the two essential regions for the replication – *oriV* and the *trfA* replication initiation protein – which seem to play an important role for the copy-number control of the RK2 plasmid [40]. Different single mutations in the *trfA* gene, causing changes in the amino acid sequence, resulted in up to 24-fold elevated plasmid copy-numbers [97]. The mutations may have affected the activity of the trfA protein and its

ability to bind to the iterons in the *oriV* sequence. Beside changes in the *trfA* gene also effects of mutations and insertions in the *oriV* sequence were investigated. As already described in the *Introduction* (see Figure 2) the *oriV* sequence contains 8 iterons (5 iterons in the small 393 bp *oriV* fragment) for the binding of the trfA initiation protein, four DnaA binding boxes for the binding of the DnaA protein, an AT-rich and a GC-rich region. Insertions of 6-11 bp between the DnaA boxes and the iterons, between the iterons and the AT-rich region and within the AT-rich region inactivated or greatly reduced the replication activity, whereas an insertion or even the deletion of the GC-rich region had little to no effect on the replication system [98]. Furthermore, for the AT-rich region also the effects of single mutations in the sequence were investigated. The AT-rich region serves as the initial site of helix destabilization and through mutations the open strand formation is affected, resulting in the inactivation of the origin [99], [100].

Regarding the single mutations in our sequence only the mismatch at position 180/347 (oriV393/oriV632) is located between two iterons sequences, which may influence the replication. The second mutation is located in the GC-rich region, which should have no effect on the replication [98] and the additional mutation in the oriV632 sequence is located upstream of the first three iterons. However, through NCBI Blast search several derivates of the RK2 plasmid were found, harboring the same sequence mutations like our two origin sequences. Comparing the different plasmids with the origin of replication of the RK2 plasmid it seems that two versions of the sequence exist. The pRK290 plasmid [91], a smaller derivate of the RK2 plasmid, containing the same sequence mutations like our two origins, is able to replicate in many gram-negative bacteria and was already applied several times in *R. eutropha* [94–96]. Comparing our oriV632 sequence to the *oriV* sequence used in the pRK290 plasmid all necessary described parts of the *oriV* region (iterons, DnaA boxes, AT-rich and GC-rich region) are identical. The only difference is that the *oriV* sequence from the pRK290 contained around 70 additional bases, located far upstream from the first iterons (shown in *Appendix 3: Sequences*). However, no functions for the additional part of the sequence are described and the origin of the RK2 plasmid was originally described as a 617 bp fragment [39]. We assumed that the sequence differences of our oriV393 and oriV632 sequence compared to the original RK2 sequence should have no effect on the replication activity and cloned

both sequences into our expression vectors. Both vectors were able to replicate in *E. coli* Top10 and R. *eutropha* H16.

During the first attempts using the new designed expression vector, containing the mini-replicon of the pBBR1 broad-host plasmid some stability and maintenance problems in *E. coli* Top 10 as well as in *R. eutropha* H 16 occurred (data not shown). Low and medium copy number plasmids often need stabilizing partitioning systems based on active partitioning, multimer resolution or on postsegregational killing [72]. Therefore the partitioning system from the RK2 broad-host plasmid, the *par* region, which was already proven to work in several gram-negative bacteria [74], was directly amplified from RK2 and cloned in all expression vectors. After the transformation of the vectors in *E.coli* Top10 cells all origins proved their functionality and via the green fluorescing colonies the expression of eGFP could be detected. The new designed vectors were then transformed into *R. eutropha* H16 via electro-transformation. Due to the presence of mobilization genes in the RSF1010-mini-replicon the pKR-Tac-Par-eGFPΔlacI RSF1010 vector was transferred into *R. eutropha* H16 via electro-transformation and via conjugation. For the determination of the plasmid replication and stable maintenance in *E. coli* Top10 and *R. eutropha* H16 the strains generated via electro-transformation were used.

Regarding the general results of the plasmid stability assays it is clearly visible that all new designed pKR-Tac-Par-eGFPΔlacI vectors with the four different origins (REP, oriV393, oriV632 and RSF1010) were 100% stable in *E. coli* Top10 as well as in *R. eutropha* H16 for the time period of 96 h. During the 96 h about 15-16 generations (equal to an average generation time of 6.3-6.4 h) in *E. coli* Top10 and 16-18 generation (equal to an average generation time of 4.5-5.9 h) in *R. eutropha* H16 were passed. Comparing the results of the *E. coli* Top10 strains the reached numbers of generations in 96 h are very similar for all different vectors. The different origins of replication seem to have no effect on the growth rate of the *E. coli* Top10 strains. Among the *R. eutropha* H16 strains with the four different vectors the strain containing the pKR-Tac-Par-eGFPΔlacI oriV393 vector achieved the lowest generation time. For the strains with the other origins the generation time was 1.3 times higher.

In general it has to be mentioned that the generation times determined for both organisms were quite high compared to the wild types. For the *R. eutropha* H16 wild type a doubling time of 3 to 4 h was measured in TSB medium [101] and the doubling time of *E. coli* Top10 wild type is only 20-40 min [102]. It is known that the maintenance and replication of plasmids and expression vectors imposes metabolic stress on *E.coli* cells which is often associated with reduced growth rates and increased doubling times [103]. However, the rather high doubling time in *E.coli* Top10 led to the assumption that the expression of all *par* genes as well as the expression of eGFP seem to be a huge challenge for the cells

However, the 100% stable maintenance and functional replication of all designed expression vectors in both organisms could be successfully shown.

In order to proof the functionality of our expression systems in *R. eutropha* H16 and to determine possible differences in the expression levels depending on the applied origin of replication the expression of eGFP was monitored via fluorescence microscopy, SDS-PAGE, Western Blot analysis and the measurement of the fluorescing units.

The *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] strain showed strong fluorescence signals during fluorescence microscopy as well as intense bands on the SDS-PAGE gel and the corresponding Western Blot. Furthermore, high amounts of relative fluorescing units of ~120 were measured for the strain containing the REP replication system. These values were comparable to already measured RFUs in *R. eutropha* H16 mentioned in literature [104]. For the *R. eutropha* H16 strain containing the expression vector with the oriV632 replicon strong fluorescence signals during microscopy and high amounts of expressed eGFP in the SDS-PAGE and the Western Blot analysis were detected. Only the relative fluorescing units of ~70 were lower, compared to the units of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] strain. The *R. eutropha* H16 strain with the smaller variant of the RK2 mini-replicon, oriV393, expressed less eGFP due to weaker fluorescing signals during microscopy and a less intense band on the SDS-PAGE gel on the corresponding Western Blot. Although the measured fluorescing units of 101 were higher as for the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI oriV632] strain we assumed that the eGFP expression work better with the vector containing the long variant of the RK2 origin. During the measurement of the fluorescing units high background

values of the negative control as well as a slight dependence of the measured FUS on the OD_{600} occurred. Therefore, more importance was attached to the results of the fluorescence microscopy and SDS-PAGE and Western Blot analysis.

Comparing the results of the eGFP expression of *R. eutropha* strains containing the two mini-replicons of the RK2 plasmid to the results of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] strain the latter achieved the highest amounts of eGFP. This could be an effect of the higher copy number of the REP origin of replication compared to the RK2 mini-replicons [23], [32]. If more plasmid copies are present in the cells higher levels of eGFP are produced. The REP origin from the pBBR1 plasmid, whose several derivates have proved to work successfully in *R. eutropha* H16 [18], [27–29], seems to be a suitable replication system for our expression vectors.

For the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] strain we expected even higher levels of expressed GFP than for the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI REP] strain, due to the higher copy number of the RSF1010 mini-replicon. However, for the *R. eutropha* H16 strain, in which the pKR-Tac-Par-eGFPΔeGFP RSF1010 vector was introduced via electro-transformation, the expression of eGFP was only detected via weak fluorescence signals collected during microscopy. Unfortunately no positive SDS-PAGE gel or Western Blot could be performed.

Considering the results of the different detection methods for eGFP, it has to be mentioned that after the electro-transformation of the vectors in *R. eutropha* H16 just a few transformants showed expression of eGFP in general. In most transformants the level of expressed eGFP was too low for any detection method. Some transformants showed fluorescence under the microscope, but no positive eGFP bands were visible on the SDS-PAGE gels or Western Blots, even after strong concentration of the samples. Furthermore, during microscope fluorescence not all cells visible in the transmitted light showed a signal in the fluorescing light. Via the comparison of the visible and the fluorescence light images for all *R. eutropha* H16 strains some cells could be identified which did not express eGFP. Therefore, a comparison of the different replication systems and the effects of their different copy numbers on the eGFP expression were rather difficult. It seemed that all *R. eutropha* H16 strains

with the different expression vectors introduced via electro-transformation had some problems with the expression of eGFP.

In order to examine this assumption the pKR-Tac-Par-eGFPΔlacI RSF1010 vector, containing the necessary mobilization genes in the RSF1010 mini-replicon, was also transferred into *R. eutropha* H16 via conjugation. The new *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] strain showed high levels of eGFP with all applied detection methods – fluorescence microscopy, SDS-PAGE and Western Blot analysis. During fluorescence microscopy all cells were strongly fluorescing and expressed high amounts of eGFP. The expressed levels of eGFP of the *R. eutropha* H16 strain, monitored via SDS-PAGE and Western Blot analysis, seemed to be as high as for the *E.coli* [pKR-Tac-Par-eGFPΔlacI RSF1010] positive control. Comparing all these positive results to the results of the *R. eutropha* H16 strain with the same vector introduced via electro-transformation the reason for the low eGFP expression levels have to be connected with the transformation process.

Although the vector has to be transferred into *R. eutropha* H16 during electro-transformation, which is proven via the selection of the transformants with TSB plates containing 200 µg/mL of kanamycin, some problems seem to occur afterwards. Considering the high generation times of the *R. eutropha* H16 strains of 4.5-6 h (see *Table 22*) the incubation time of 2 h after the transformation may be too short for the regeneration of the cells and a proper replication of the plasmid. During the conjugation process the donor strain and the recipient strain are incubated overnight without any selective pressure. Hence, the cells have more time to regenerate and to replicate the vector before they are put under selective pressure.

In literature only a few studies, using electro-transformation in *R. eutropha* H16 could be found. However, one study described the successful transformation of the pKT230 plasmid into *R. eutropha* H16, applying the same protocol like our group [105], [106]. The transformed plasmid is a derivate of the RSF1010 broad-host-range plasmid and contains the same RSF1010 mini-replicon we used in our expression vector [107]. No problems considering the replication of the plasmid or negative effects on the copy number and the expression of desired genes were reported in this study [106].

However, comparing the amount of performed studies with *R. eutropha* H16 with regard to the applied method of plasmid transfer conjugation was the predominant method used. Several studies conjugating expression vectors with the REP origin of replication [18], [29], [108], the origin of the RK2 plasmid [109], [110] and also the RSF1010 origin [13], [52], [111] were already performed. Although no studies comparing the advantages of the conjugation against electro-transformation in *R. eutropha* H16 could be found it seems that conjugation is the more popular method.

Application of lacZ as reporter gene

Due to the weak fluorescing signal of eGFP and some detection problems *lacZ* was tested as alternative reporter gene to *egfp*. In literature many studies are described where *lacZ* was used successfully as reporter gene in *R. eutropha* H16 [21], [112–116]. The expression of the *lacZ* gene in *R. eutropha* H16 strains was detected via the β-galactosidase assay. Several transformants were tested for the presence of β-galactosidase but no yellow color change indicating the enzymatic conversion of oNPG to oNP was observed. As second approach for the detection of LacZ protein SDS-PAGE was performed. However, no LacZ band could be detected either (data not shown).

Although the presence of the *lacZ* gene in the analyzed *R. eutropha* H16 strains was demonstrated by PCR, no LacZ expression could be detected, applying the standard detection methods like the β-galactosidase assay or SDS-PAGE. Even though *lacZ* was applied as reporter gene in *R. eutropha* H16 in several studies [21], [112], [113] the combination of *lacZ* and the *par* region in our expression vector may have caused too much stress for the cells leading to an interruption of the LacZ expression. Regarding also the mentioned problems during the expression of eGFP another reason would be that the expression levels of LacZ were just too low for the detection via the β-galactosidase assay. For cell lysis of the *R. eutropha* H16 samples the double amount of chloroform and SDS had to be applied compared to *E. coli* cells [89]. The high levels of SDS may lead to the denaturation of small amounts of β-galactosidase.

Due to the detection problems of *lacZ* in *R. eutropha* H16 during our work we continued with the application of *eGFP* as reporter gene for our expression vectors.

Conclusion and Outlook

In this work four new expression vectors for *R. eutropha* H16 containing different origins of replication – the REP and RSF1010 from the medium copy number plasmid pBBR1 and the rather high copy number plasmid RSF1010, respectively, as well as the two variants oriV393 and oriV632 from the low copy number plasmid RK2 – were constructed. With the partitioning system from the RK2 plasmid, the *par* region, the 100% stability and stable maintenance of all vectors in the *R. eutropha* H16 strains was achieved. The expression of the applied reporter protein eGFP could be detected for all strains, although some problems occurred. Some clones did not express eGFP at all and during fluorescence microscopy many cells could be detected, which contained the plasmid but were not expressing eGFP. Therefore, a comparison of the different replication systems and a proposition of possible effects of the different copy numbers on the eGFP expression were rather difficult.

Via the generation of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] strain by the two different transfer methods – electro-transformation and conjugation – it could be shown that the problems with the eGFP expression seemed to be connected with the electro-transformation. During fluorescence microscopy of the *R. eutropha* H16 [pKR-Tac-Par-eGFPΔlacI RSF1010] generated via conjugation all cells showed high levels of expressed eGFP. The conjugation seems to be the better method for plasmid transfer into the *R. eutropha* H16 cells, resulting in a much higher eGFP expression.

For future studies it would be very interesting to further analyze the problems which apparently occurred during the electro-transformation and to compare the different effects of the transformation and the conjugation in *R. eutropha* H16 on the protein expression level. For the conjugation of the other designed expression vectors – containing the REP origin of replication, the oriV393 and oriV632 – the necessary mobilization genes have to be cloned into the vectors. After the successful conjugation of all expression vectors in *R. eutropha* H16 a new comparison of the different replication systems could be performed, analyzing possible effects of their different copy numbers on the protein expression.

Furthermore, in order to achieve higher expression levels and to reduce the stress on the cells at the same time, the expression phase could be separated from the growth phase via the generation of an inducible expression system for *R. eutropha* H16. The incapability to assimilate lactose or IPTG for the application of the inducible Lac promoter system could be overcome by the introduction of the *lacY* gene encoding a lactose permease. This would provide an inducible expression system for *R. eutropha* H16, which could be applied also during different growth conditions.

References

[1] H. G. Aragno, M.; Schlegel, “The mesophilic hydrogen-oxidizing (knallgas) bacterium.,” in *The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*, 1st ed., K. H. Balows, A.;Truper, H.G.;Dworkin, M.;Harder, W.;Schleifer, Ed. New York, Springer, 1992, pp. 344–384.

[2] B. Fricke, W. F.·Kusian, B. Bowien, “The Genome Organization of Ralstonia eutropha Strain H16 and Related Species of the Burkholderiaceae -,” *Jounral of molecular microbiology and biotechnology*, vol. 16, no. 1–2, pp. 124–135, 2009.

[3] B. Friedrich and E. Schwartz, “Molecular biology of hydrogen utilization in aerobic chemolithotrophs.,” *Annual review of microbiology*, vol. 47, pp. 351–83, Jan. 1993.

[4] D. Römermann and B. Friedrich, “Denitrification by Alcaligenes eutrophus Is Plasmid Dependent,” *Journal of Bacteriology*, vol. 162, no. 2, pp. 852–854, 1985.

[5] A. Steinbüchel and B. Füchtenbusch, “Bacterial and other biological systems for polyester production,” *Trends in Biotechnology*, vol. 16, no. 10, pp. 419–427, Oct. 1998.

[6] A. Pohlmann, W. F. Fricke, F. Reinecke, B. Kusian, H. Liesegang, R. Cramm, T. Eitinger, C. Ewering, M. Pötter, E. Schwartz, A. Strittmatter, I. Voss, G. Gottschalk, A. Steinbüchel, B. Friedrich, and B. Bowien, “Genome sequence of the bioplastic-producing ‘Knallgas’ bacterium Ralstonia eutropha H16.,” *Nature Biotechnology*, vol. 24, no. 10, pp. 1257–1262, Oct. 2006.

[7] E. Schwartz, A. Henne, R. Cramm, T. Eitinger, B. Friedrich, and G. Gottschalk, “Complete Nucleotide Sequence of pHG1: A Ralstonia eutropha H16 Megaplasmid Encoding Key Enzymes of H2-based Lithoautotrophy and Anaerobiosis,” *Journal of Molecular Biology*, vol. 332, pp. 369–383, 2003.

[8] E. Wilde, “Untersuchungen über Wachstum und Speicherstoffsynthese von Hydrogenomonas,” *Archiv für Mikrobiologie*, vol. 43, no. 2, pp. 109–137, 1962.

[9] C. König, I. Sammler, E. Wilde, and H. G. Schlegel, “Konstitutive Glucose-6-phosphat-Dehydrogenase bei Glucose verwertenden Mutanten von einem kryptischen Wildstamm,” *Archiv für Mikrobiologie*, vol. 67, no. 1, pp. 51–57, 1969.

[10] R. Cramm, R. a Siddiqui, and B. Friedrich, “Two isofunctional nitric oxide reductases in Alcaligenes eutrophus H16.,” *Journal of bacteriology*, vol. 179, no. 21, pp. 6769–77, Nov. 1997.

[11] B. Bowien and H. G. Schlegel, “Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria.,” *Annual review of microbiology*, vol. 35, pp. 405–52, Jan. 1981.

[12] a Tran-Betcke, U. Warnecke, C. Böcker, C. Zaborosch, and B. Friedrich, “Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of Alcaligenes eutrophus H16.,” *Journal of bacteriology*, vol. 172, no. 6, pp. 2920–9, Jun. 1990.

[13] C. Kortlüke, K. Horstmann, E. Schwartz, M. Rohde, R. Binsack, and B. Friedrich, “A gene complex coding for the membrane-bound hydrogenase of Alcaligenes eutrophus H16.,” *Journal of bacteriology*, vol. 174, no. 19, pp. 6277–89, Oct. 1992.

[14] B. Kusian and B. Bowien, “Organization and regulation of cbb CO2 assimilation genes in autotrophic bacteria.,” *FEMS microbiology reviews*, vol. 21, no. 2, pp. 135–55, Sep. 1997.

[15] S. J. Lee, S. Y., Park, “Fermentative production of short-chain- length PHAs,” in *Biotechnology of biopolymers: from synthesis to patents*, A. Steinbüchel and Y. Dio, Eds. Wiley-VCH, Weinheim, German, 2005, pp. 207–234.

[16] M. E. Kovach, P. H. Elzer, D. S. Hill, G. T. Robertson, M. a Farris, R. M. Roop, and K. M. Peterson, “Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes.,” *Gene*, vol. 166, no. 1. Gene, pp. 175–176, 1995.

[17] S. Sichwart, S. Hetzler, D. Bröker, and A. Steinbüchel, “Extension of the substrate utilization range of Ralstonia eutropha strain H16 by metabolic engineering to include mannose and glucose.,” *Applied and environmental microbiology*, vol. 77, no. 4, pp. 1325–34, Feb. 2011.

[18] T. Fukui, K. Ohsawa, J. Mifune, I. Orita, and S. Nakamura, “Evaluation of promoters for gene expression in polyhydroxyalkanoate-producing Cupriavidus necator H16.,” *Applied microbiology and biotechnology*, vol. 89, no. 5, pp. 1527–36, Mar. 2011.

[19] R. Cramm, A. Büsch, and K. Strube, “NO-dependent transcriptional activation of gene expression in Ralstonia eutropha H16.,” *Biochemical Society transactions*, vol. 34, no. Pt 1, pp. 182–4, Feb. 2006.

[20] O. Lenz, A. Strack, A. Tran-Betcke, and B. Friedrich, “A hydrogen-sensing system in transcriptional regulation of hydrogenase gene expression in Alcaligenes species.,” *Journal of bacteriology*, vol. 179, no. 5, pp. 1655–63, Mar. 1997.

[21] J. Hänisch, M. Wältermann, H. Robenek, and A. Steinbüchel, “The Ralstonia eutropha H16 phasin PhaP1 is targeted to intracellular triacylglycerol inclusions in Rhodococcus opacus PD630 and Mycobacterium smegmatis mc2155, and provides an anchor to target other proteins.,” *Microbiology*, vol. 152, no. Pt 11, pp. 3271–80, Nov. 2006.

[22] A. Jesu, R. Giraldo, and N. Di, “Replication and Control of Circular Bacterial Plasmids,” *Microbiology and Molculare Biology Reviews*, vol. 62, no. 2, pp. 434–464, 1998.

[23] R. Antoine and C. Locht, “Isolation and molecular characterization of a novel broad-host-range plasmid from Bordetella bronchiseptica with sequence similarities to plasmids from Gram-positive organisms,” *Molecular Microbiology*, vol. 6, no. 13, pp. 1785–1799, Jul. 1992.

[24] M. E. Kovach, R. W. Phillips, P. H. Elzer, R. M. Roop, and K. M. Peterson, “pBBR1MCS: a broad-host-range cloning vector.,” *BioTechniques*, vol. 16, no. 5, pp. 800–2, May 1994.

[25] P. H. Elzer, M. E. Kovach, R. W. Phillips, G. T. Robertson, K. M. Peterson, and R. M. Roop, “In vivo and in vitro stability of the broad-host-range cloning vector pBBR1MCS in six Brucella species.,” *Plasmid*, vol. 33, no. 1. pp. 51–57, 1995.

[26] C. Y. Szpirer, M. Faelen, and M. Couturier, “Mobilization function of the pBHR1 plasmid, a derivative of the broad-host-range plasmid pBBR1.,” *Journal Of Bacteriology*, vol. 183, no. 6, pp. 2101–2110, 2001.

[27] C. Ewering, T. Lütke-Eversloh, H. Luftmann, and A. Steinbüchel, “Identification of novel sulfur-containing bacterial polyesters: biosynthesis of poly(3-hydroxy-S-propyl-omega-thioalkanoates) containing thioether linkages in the side chains.,” *Microbiology (Reading, England)*, vol. 148, no. Pt 5, pp. 1397–406, May 2002.

[28] M. V Coppi, C. Leang, S. J. Sandler, and D. R. Lovley, “Development of a genetic system for Geobacter sulfurreducens.,” *Applied and environmental microbiology*, vol. 67, no. 7, pp. 3180–7, Jul. 2001.

[29] J. Overhage, A. Steinbüchel, and H. Priefert, “Biotransformation of eugenol to ferulic acid by a recombinant strain of Ralstonia eutropha H16.,” *Applied and environmental microbiology*, vol. 68, no. 9, pp. 4315–21, Sep. 2002.

[30] T. C. M. Pansegrau W., Lanka E., Barth P.t., Figurski D.H., Guiny D.G., Haas D., Helsinki D.R., Schwab H., Stanisich V.A., "Complete Nucleotide Sequence of Birmingham IncPα Plasmids: Compilation and Comparative Analysis," *Jounral of Molecular Biotechnology*, vol. 239, no. 5, pp. 623–663, 1994.

[31] G. K. Chikami, D. G. Guiney, T. J. Schmidhauser, and D. R. Helinski, "Comparison of 10 IncP plasmids: homology in the regions involved in plasmid replication.," *Journal of bacteriology*, vol. 162, no. 2, pp. 656–60, May 1985.

[32] D. Thomas, C., Helinski, "Vegetative replication and stable inheritance of IncP plsamids," in *Promiscuous plasmids of Gram-negative Bacteria*, T. C.M., Ed. Academic Press Inc. Ltd, London, 1989, pp. 1–25.

[33] K. Kolatka, S. Kubik, M. Rajewska, and I. Konieczny, "Replication and partitioning of the broad-host-range plasmid RK2.," *Plasmid*, vol. 64, no. 3, pp. 119–34, Nov. 2010.

[34] C. M. Thomas, C. A. Smith, V. Shingler, M. A. Cross, A. A. Hussain, and M. Pinkney, "Regulation of replication and maintenance functions of broad host-range plasmid RK2.," *Basic life sciences*, vol. 30, pp. 261–76, Jan. 1985.

[35] A. Toukdarian, "Plasmid Strategies for Broad-Host-Range Replication in Gram-Negatve Bacteria," in *Plasmid Biology*, G. J. Funnell, B. E., Phillips, Ed. ASM Press, Washington DC, USA, 2004, pp. 259–270.

[36] C. M. Thomas and A. A. Hussain, "The korB gene of broad host range plasmid RK2 is a major copy number control element which may act together with trfB by limiting trfA expression.," *The EMBO journal*, vol. 3, no. 7, pp. 1513–9, Jul. 1984.

[37] M. a Cross, S. R. Warne, and C. M. Thomas, "Analysis of the vegetative replication origin of broad-host-range plasmid RK2 by transposon mutagenesis.," *Plasmid*, vol. 15, no. 2, pp. 132–46, Mar. 1986.

[38] C. M. Thomas, "Complementation analysis of replication and maintenance functions of broad host range plasmids RK2 and RP1," *Plasmid*, vol. 5, no. 3, pp. 277–291, May 1981.

[39] D. M. Stalker, C. M. Thomas, and D. R. Helinski, "Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2.," *Molecular & general genetics : MGG*, vol. 181, no. 1, pp. 8–12, Jan. 1981.

[40] B. L. Kittell and D. R. Helinski, "Iteron inhibition of plasmid RK2 replication in vitro: evidence for intermolecular coupling of replication origins as a mechanism for RK2 replication control.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 4, pp. 1389–93, Feb. 1991.

[41] V. Shingler and C. M. Thomas, "Analysis of the trfA region of broad host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products.," *Journal of molecular biology*, vol. 175, no. 3, pp. 229–49, May 1984.

[42] J. A. Kornacki, A. H. West, and W. Firshein, "Proteins encoded by the trans-acting replication and maintenance regions of broad host range plasmid RK2.," *Plasmid*, vol. 11, no. 1, pp. 48–57, Jan. 1984.

[43] S. Perri, D. R. Helinski, and a Toukdarian, "Interactions of plasmid-encoded replication initiation proteins with the origin of DNA replication in the broad host range plasmid RK2.," *The Journal of biological chemistry*, vol. 266, no. 19, pp. 12536–43, Jul. 1991.

[44] F. C. Fang and D. R. Helinski, "Broad-host-range properties of plasmid RK2: importance of overlapping genes encoding the plasmid replication initiation protein TrfA.," *Journal of bacteriology*, vol. 173, no. 18, pp. 5861–8, Sep. 1991.

[45] C. M. Thomas, D. M. Stalker, and D. R. Helinski, “Replication and incompatibility properties of segments of the origin region of replication of the broad host range plasmid RK2.,” *Molecular & general genetics*, vol. 181, no. 1, pp. 1–7, Jan. 1981.

[46] H. Schwab, P. N. Saurugger, and R. M. Lafferty, “Occurrence of deletion plasmids at high rates after conjugative transfer of the plasmids RP4 and RK2 from Escherichia coli to Alcaligenes eutrophus H16.,” *Archives of microbiology*, vol. 136, no. 2, pp. 140–6, Nov. 1983.

[47] C. M. Thomas, M. a Cross, a a Hussain, and C. a Smith, “Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2.,” *The EMBO journal*, vol. 3, no. 1, pp. 57–63, Jan. 1984.

[48] T. J. Schmidhauser, M. Filutowicz, and D. R. Helinski, “Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria.,” *Plasmid*, vol. 9, no. 3, pp. 325–30, May 1983.

[49] and M. B. Bagdasarian, M. M., P. Scholz, J. Frey, “Regulation of the rep operon expression in the broad host range,” *Banbury Report*, vol. 24, pp. 209–224, 1986.

[50] U. Kües and U. Stahl, “Replication of plasmids in gram-negative bacteria.,” *Microbiological reviews*, vol. 53, no. 4, pp. 491–516, 1989.

[51] M. Bagdasarian, R. Lurz, B. Ruckert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis, “Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas,” *Gene*, vol. 16, no. 1–3. pp. 237–247, 1981.

[52] T. Fukui and Y. Doi, “Efficient production of polyhydroxyalkanoates from plant oils by Alcaligenes eutrophus and its recombinant strain,” *Applied Microbiology and Biotechnology*, vol. 49, no. 3, pp. 333–336, Mar. 1998.

[53] M. A. Brasch and R. J. Meyer, “Genetic organization of plasmid R1162 DNA involved in conjugative mobilization.,” *Journal of bacteriology*, vol. 167, no. 2, pp. 703–10, Aug. 1986.

[54] L. S. Lin and R. J. Meyer, “Directly repeated, 20-bp sequence of plasmid R1162 DNA is required for replication, expression of incompatibility, and copy-number control.,” *Plasmid*, vol. 15, no. 1, pp. 35–47, Jan. 1986.

[55] L. S. Lin and R. J. Meyer, “Nucleotide sequence and functional properties of DNA encoding incompatibility in the broad host-range plasmid R1162.,” *Molecular & general genetics : MGG*, vol. 194, no. 3, pp. 423–31, Jan. 1984.

[56] L. S. Lin and R. J. Meyer, “DNA synthesis is initiated at two positions within the origin of replication of plasmid R1162.,” *Nucleic acids research*, vol. 15, no. 20, pp. 8319–31, Oct. 1987.

[57] E. Scherzinger, M. M. Bagdasarian, P. Scholz, R. Lurz, B. Rückert, and M. Bagdasarian, “Replication of the broad host range plasmid RSF1010: requirement for three plasmid-encoded proteins.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 3, pp. 654–658, 1984.

[58] V. Haring, P. Scholz, E. Scherzinger, J. Frey, K. Derbyshire, G. Hatfull, N. S. Willetts, and M. Bagdasarian, “Protein RepC is involved in copy number control of the broad host range plasmid RSF1010.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 18, pp. 6090–4, Sep. 1985.

[59] J. Bagdasarian, M., Bagdasarian, M.M., Lurz, R., Nordhcim. A., Frey and K. N. Timmis, “Molecular and functional analysis of the broadhost- Gene, range plasmid RSFI010 and construction of vectors for cloning in Gram-negative bacteria.,” in *Bacterial Drug Resistance*, S. Mitsuhashi, Ed. Japan Science Society, Press, Tokyo, 1982, pp. 183–197.

[60] V. Bacteriology and B. Laenggasstr, “Replication and copy number control of the broad-host-range plasmid RSFI010 Joachim Frey a , Mira M . Bagdasarian b and Michael Bagdasarian b,” vol. 113, pp. 101–106, 1992.

[61] P. Scholz, V. Haring, B. Wittmann-liehold, K. Ashman, and M. Bagdasarian, “Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010.,” *Gene*, vol. 75, no. 2, pp. 271–288, 1989.

[62] K. M. Derbyshire, G. Hatfull, and N. Willetts, “Mobilization of the non-conjugative plasmid RSF1010: a genetic and DNA sequence analysis of the mobilization region.,” *Molecular & general genetics : MGG*, vol. 206, no. 1, pp. 161–8, Jan. 1987.

[63] M. A. Brasch and R. J. Meyer, “A 38 base-pair segment of DNA is required in cis for conjugative mobilization of broad host-range plasmid R1162.,” *Journal of molecular biology*, vol. 198, no. 3, pp. 361–9, Dec. 1987.

[64] R. J. Kim, K., Meyer, “Unidirectional transfer of broad host-range plasmid RII 62 during conjugative mobilization. Evidence for genetically distinct events at oriT.,” *Journal of Molecular Biology*, vol. 208, pp. 501–508, 1989.

[65] E. Scherzinger, R. Lurz, S. Otto, and B. Dobrinski, “In vitro cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins.,” *Nucleic acids research*, vol. 20, no. 1, pp. 41–8, Jan. 1992.

[66] M. Drolet, P. Zanga, and P. C. K. Lau, “The mobilization and origin of transfer regions of a Thiobacillus ferrooxidans plasmid: relatedness to plasmids RSF1010 and pSC101,” *Molecular Microbiology*, vol. 4, no. 8, pp. 1381–1391, Aug. 1990.

[67] R. Simon, U. Priefer, and A. Pühler, “A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria,” *Bio/Technology*, vol. 1, no. 9, pp. 784–791, Nov. 1983.

[68] P. Schubert, A. Steinbuchel, and H. G. Schlegel, “Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli.,” *J. Bacteriol.*, vol. 170, no. 12, pp. 5837–5847, Dec. 1988.

[69] E. Top, M. Mergeay, D. Springael, and W. Verstraete, “Gene escape model: transfer of heavy metal resistance genes from Escherichia coli to Alcaligenes eutrophus on agar plates and in soil samples.,” *Appl. Envir. Microbiol.*, vol. 56, no. 8, pp. 2471–2479, Aug. 1990.

[70] O. Lenz, E. Schwartz, J. Dernedde, M. Eitinger, and B. Friedrich, “The Alcaligenes eutrophus H16 hoxX gene participates in hydrogenase regulation.,” *J. Bacteriol.*, vol. 176, no. 14, pp. 4385–4393, Jul. 1994.

[71] R. A. Siddiqui, U. Warnecke-Eberz, A. Hengsberger, B. Schneider, S. Kostka, and B. Friedrich, “Structure and function of a periplasmic nitrate reductase in Alcaligenes eutrophus H16.,” *J. Bacteriol.*, vol. 175, no. 18, pp. 5867–5876, Sep. 1993.

[72] U. Zielenkiewicz and P. Cegłowski, “Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems.,” *Acta biochimica Polonica*, vol. 48, no. 4, pp. 1003–23, Jan. 2001.

[73] R. P. Helinski, D. R., Toukdarian, A. E., Novick, “Replication control and other stable maintenance mechanisms of plasmids,” in *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed., F. C. et al Niedhardt, Ed. American Society for Microbiology, Washington, D.C., 1996, pp. 2295–2324.

[74] P. N. Saurugger, O. Hrabak, H. Schwab, and R. M. Lafferty, “Mapping and cloning of the par-region of broad-host-range plasmid RP4,” *Journal of Biotechnology*, vol. 4, no. 6, pp. 333–343, Dec. 1986.

[75] P. a Sobecky, C. L. Easter, P. D. Bear, and D. R. Helinski, “Characterization of the stable maintenance properties of the par region of broad-host-range plasmid RK2.,” *Journal Of Bacteriology*, vol. 178, no. 7, pp. 2086–2093, 1996.

[76] L. Eberl, C. S. Kristensen, M. Givskov, E. Grohmann, M. Gerlitz, and H. Schwab, “Analysis of the multimer resolution system encoded by the parCBA operon of broad-host-range plasmid RP4,” *Molecular Microbiology*, vol. 12, no. 1, pp. 131–141, Apr. 1994.

[77] E. P. Johnson, a R. Strom, and D. R. Helinski, “Plasmid RK2 toxin protein ParE: purification and interaction with the ParD antitoxin protein.,” *Journal of bacteriology*, vol. 178, no. 5, pp. 1420–9, Mar. 1996.

[78] J. Hänisch, M. Wältermann, H. Robenek, and A. Steinbüchel, “The Ralstonia eutropha H16 phasin PhaP1 is targeted to intracellular triacylglycerol inclusions in Rhodococcus opacus PD630 and Mycobacterium smegmatis mc2155, and provides an anchor to target other proteins.,” *Microbiology*, vol. 152, no. Pt 11, pp. 3271–3280, Nov. 2006.

[79] D. Jendrossek, “Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes).,” *Journal of bacteriology*, vol. 191, no. 10, pp. 3195–202, May 2009.

[80] F. Hempel, A. S. Bozarth, N. Lindenkamp, A. Klingl, S. Zauner, U. Linne, A. Steinbüchel, and U. G. Maier, “Microalgae as bioreactors for bioplastic production.,” *Microbial cell factories*, vol. 10, p. 81, Jan. 2011.

[81] O. Shimomura, F. H. Johnson, and Y. Saiga, “Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea.,” *Journal of cellular and comparative physiology*, vol. 59, pp. 223–39, Jun. 1962.

[82] B. P. Cormack, R. H. Valdivia, and S. Falkow, “FACS-optimized mutants of the green fluorescent protein (GFP).,” *Gene*, vol. 173, no. 1 Spec No, pp. 33–8, Jan. 1996.

[83] A. G. Matthysse, S. Stretton, C. Dandie, N. C. McClure, and A. E. Goodman, “Construction of GFP vectors for use in Gram-negative bacteria other than Escherichia coli,” *FEMS Microbiology Letters*, vol. 145, no. 1, pp. 87–94, Nov. 1996.

[84] S. Sichwart, S. Hetzler, D. Bröker, and A. Steinbüchel, “Extension of the substrate utilization range of Ralstonia eutropha strain H16 by metabolic engineering to include mannose and glucose.,” *Applied and environmental microbiology*, vol. 77, no. 4, pp. 1325–34, Feb. 2011.

[85] M. Gerlitz, O. Hrabak, and H. Schwab, “Partitioning of broad-host-range plasmid RP4 is a complex system involving site-specific recombination.,” *Journal of bacteriology*, vol. 172, no. 11, pp. 6194–203, Nov. 1990.

[86] H. G. Schlegel, H. Kaltwasser, and G. Gottschalk, “Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen,” *Archiv für Mikrobiologie*, vol. 38, no. 3, pp. 209–222, 1961.

[87] T. J. Schmidhauser and D. R. Helinski, “Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria.,” *Journal of bacteriology*, vol. 164, no. 1, pp. 446–55, Oct. 1985.

[88] A. Pries, A. Steinbüchel, and H. Schlegel, “Lactose- and galactose-utilizing strains of poly(hydroxyalkanoic acid)-accumulating Alcaligenes eutrophus and Pseudomonas saccharophila obtained by recombinant DNA technology,” *Applied Microbiology and Biotechnology*, vol. 33, no. 4, Jul. 1990.

[89] H. Li, P. H. Opgenorth, D. G. Wernick, S. Rogers, T.-Y. Wu, W. Higashide, P. Malati, Y.-X. Huo, K. M. Cho, and J. C. Liao, "Integrated electromicrobial conversion of CO2 to higher alcohols.," *Science (New York, N.Y.)*, vol. 335, no. 6076, p. 1596, Mar. 2012.

[90] A. S. Haines, K. Jones, S. M. Batt, I. a Kosheleva, and C. M. Thomas, "Sequence of plasmid pBS228 and reconstruction of the IncP-1alpha phylogeny.," *Plasmid*, vol. 58, no. 1, pp. 76–83, Jul. 2007.

[91] G. Ditta, T. Schmidhauser, E. Yakobson, P. Lu, X. W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski, "Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression.," *Plasmid*, vol. 13, no. 2, pp. 149–53, Mar. 1985.

[92] C. J. Marx and M. E. Lidstrom, "Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria.," *Microbiology (Reading, England)*, vol. 147, no. Pt 8, pp. 2065–75, Aug. 2001.

[93] H. N. Scott, P. D. Laible, and D. K. Hanson, "Sequences of versatile broad-host-range vectors of the RK2 family," *Plasmid*, vol. 50, no. 1, pp. 74–79, Jul. 2003.

[94] K. Andersen and M. Wilke-Douglas, "Genetic and physical mapping and expression in Pseudomonas aeruginosa of the chromosomally encoded ribulose bisphosphate carboxylase genes of Alcaligenes eutrophus.," *Journal of bacteriology*, vol. 169, no. 5, pp. 1997–2004, May 1987.

[95] N. Isolate, J. B. Utåker, K. Andersen, Å. Aakra, B. Moen, and I. F. Nes, "Phylogeny and Functional Expression of Carboxylase / Oxygenase from the Autotrophic Ammonia-Oxidizing Bacterium Phylogeny and Functional Expression of Ribulose 1 , 5-Bisphosphate Carboxylase / Oxygenase from the Autotrophic Ammonia-Oxidizing Bacterium Nit," 2002.

[96] D. Zimmer, E. Schwartz, P. Gewinner, D. Zimmer, E. Schwartz, A. Tran-betcke, and P. Gewinner, "Temperature tolerance of hydrogenase expression in Alcaligenes eutrophus is conferred by a single amino acid exchange in the transcriptional activator HoxA . Temperature Tolerance of Hydrogenase Expression in Alcaligenes eutrophus Is Conferred by a Single," 1995.

[97] R. H. Durland, a Toukdarian, F. Fang, and D. R. Helinski, "Mutations in the trfA replication gene of the broad-host-range plasmid RK2 result in elevated plasmid copy numbers.," *Journal of bacteriology*, vol. 172, no. 7, pp. 3859–67, Jul. 1990.

[98] K. S. Doran, I. Konieczny, and D. R. Helinski, "Replication Origin of the Broad Host Range Plasmid RK2," vol. 273, no. 14, pp. 8447–8453, 1998.

[99] L. Kowalczyk, M. Rajewska, and I. Konieczny, "Positioning and the specific sequence of each 13-mer motif are critical for activity of the plasmid RK2 replication origin.," *Molecular microbiology*, vol. 57, no. 5, pp. 1439–49, Sep. 2005.

[100] M. Rajewska, L. Kowalczyk, G. Konopa, and I. Konieczny, "Specific mutations within the AT-rich region of a plasmid replication origin affect either origin opening or helicase loading.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 32, pp. 11134–9, Aug. 2008.

[101] J. Tian, A. J. Sinskey, and J. Stubbe, "Kinetic Studies of Polyhydroxybutyrate Granule Formation in Wautersia eutropha H16 by Transmission Electron Microscopy," *Jounral of Bacteriology*, vol. 187, no. 11, pp. 3814–3824, 2005.

[102] M. Chandler, R. E. Bird, and L. Caro, "The replication time of the Escherichia coli K12 chromosome as a function of cell doubling time.," *Journal of molecular biology*, vol. 94, no. 1, pp. 127–32, May 1975.

[103] J. C. D. Ricci and M. E. Hernández, "Plasmid Effects on Escherichia coli Metabolism," *Critical Reviews in Biotechnology*, vol. 20, no. 2, pp. 79–108, Sep. 2000.

[104] D. C. Reed, G. C. Barnard, E. B. Anderson, L. T. Klein, and T. U. Gerngross, “Production and purification of self-assembling peptides in Ralstonia eutropha.,” *Protein expression and purification*, vol. 46, no. 2, pp. 179–88, Apr. 2006.

[105] H. Park, K. Lim, J. Park, Y. Lee, and T. Huh, “High frequency transformation of Alcaligenes eutrophus producing poly-?-hydroxybutyric acid by electroporation,” *Biotechnology Techniques*, vol. 9, no. 1, pp. 31–34, Jan. 1995.

[106] Y. Jung, J. Park, and Y. Lee, “Metabolic engineering of Alcaligenes eutrophus through the transformation of cloned phbCAB genes for the investigation of the regulatory mechanism of polyhydroxyalkanoate biosynthesis,” vol. 26, pp. 201–208, 2000.

[107] H. Franklin and M. Bagdasarian, “Molecular and functional analysis of the TOL plasmid pWWO from Pseudomonas putida and cloning of genes for the entire regulated aromatic ring meta cleavage pathway,” vol. 78, no. 12, pp. 7458–7462, 1981.

[108] K. Peplinski, A. Ehrenreich, C. Döring, M. Bömeke, F. Reinecke, C. Hutmacher, and A. Steinbüchel, “Genome-wide transcriptome analyses of the ‘Knallgas’ bacterium Ralstonia eutropha H16 with regard to polyhydroxyalkanoate metabolism.,” *Microbiology*, vol. 156, no. Pt 7, pp. 2136–2152, Jul. 2010.

[109] D. Nies, M. Mergeay, B. Friedrich, and H. G. Schlegel, “Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in Alcaligenes eutrophus CH34.,” *Journal of bacteriology*, vol. 169, no. 10, pp. 4865–8, Oct. 1987.

[110] T. H. E. Phb, P. Gene, O. P. Peoples, and A. J. Sinskey, “Poly-P-hydroxybutyrate (PHB) Biosynthesis in Alcaligenes eutrophus H16- Identification and characterization of the PHB Polymerase Gene (phbC),” *The Journal of biological chemistry*, vol. 264, no. 26, pp. 15298–15303, 1989.

[111] K. Smalla, H. Heuer, A. Götz, E. Krögerrecklenfort, and E. Tietze, “Exogenous Isolation of Antibiotic Resistance Plasmids from Piggery Manure Slurries Reveals a High Prevalence and Diversity of IncQ-Like Plasmids Three-Year Study To Assess Human Enteric Viruses in Shellfish Molecular Analysis of the Microbial Diversity Pr,” *Applied and environmental microbiology*, vol. 66, no. 11, pp. 4854–4862, 2000.

[112] A. Steinbüchel and H. G. Schlegel, “Physiology and molecular genetics of poly (p-hydroxy- alkanoic acid) synthesis in Alcaligenes eutrophus,” *Molecular Microbiology*, vol. 5, no. 3, pp. 535–542, 1991.

[113] C. Kortlüke, K. Horstmann, E. Schwartz, M. Rohde, R. Binsack, and B. Friedrich, “A gene complex coding for the membrane-bound hydrogenase of Alcaligenes eutrophus H16.,” *Journal of bacteriology*, vol. 174, no. 19, pp. 6277–89, Oct. 1992.

[114] A. Pohlmann, R. Cramm, K. Schmelz, and B. Friedrich, “A novel NO-responding regulator controls the reduction of nitric oxide in Ralstonia eutropha.,” *Molecular microbiology*, vol. 38, no. 3, pp. 626–38, Nov. 2000.

[115] O. Lenz, M. Bernhard, T. Buhrke, E. Schwartz, and B. Friedrich, “The hydrogen-sensing apparatus in Ralstonia eutropha.,” *Journal of molecular microbiology and biotechnology*, vol. 4, no. 3, pp. 255–62, May 2002.

[116] T. Schubert, O. Lenz, E. Krause, R. Volkmer, and B. Friedrich, “Chaperones specific for the membrane-bound [NiFe]-hydrogenase interact with the Tat signal peptide of the small subunit precursor in Ralstonia eutropha H16.,” *Molecular microbiology*, vol. 66, no. 2, pp. 453–67, Oct. 2007.

Appendix 1: Figures and Tables

Figures

Tables

Appendix 2: Vector maps

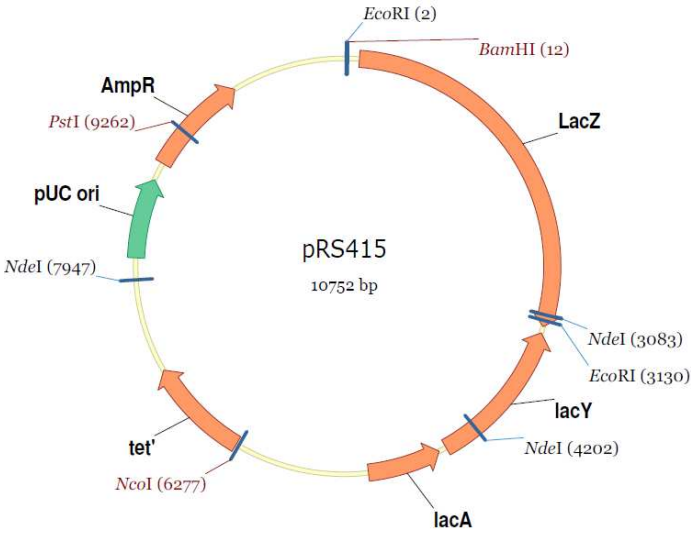


Figure 48: Vector map of pRS415, containing the pUC ori as origin of replication, the ampicillin and tetracyclin resistance genes, as well as the lac operon.

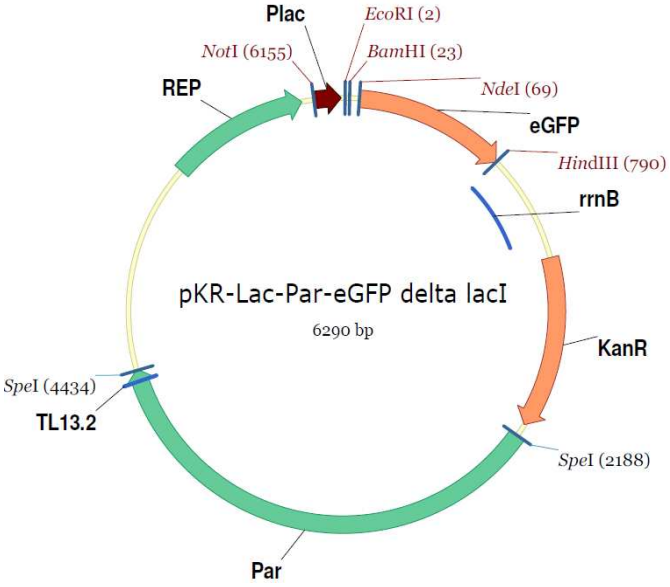


Figure 49: Vector map of pKR-Lac-Par-egfpΔlacI REP, containing the Lac promoter, the REP origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designed par sequence.

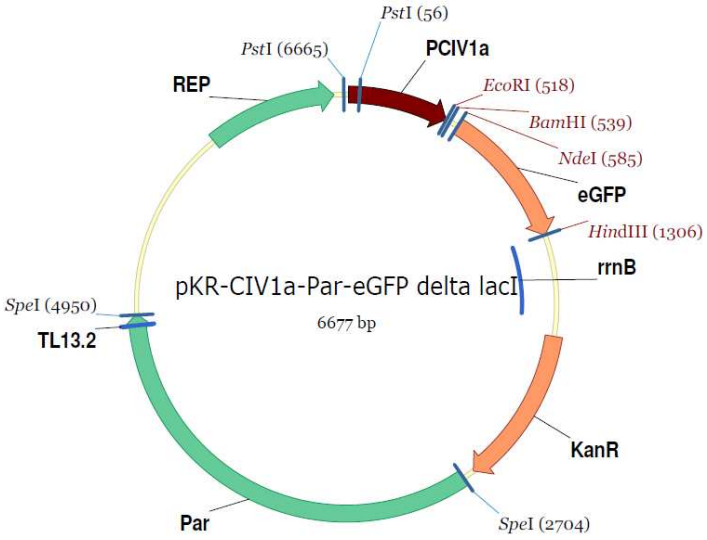


Figure 50: Vector map of pKR-CIV1a-Par-egfpΔlacI REP, containing the CIV1a promoter originating form R. eutropha H16, the REP origin of replication, the kanamycin resistance gene, egfp as reporter gene and the new designedpa -sequence.

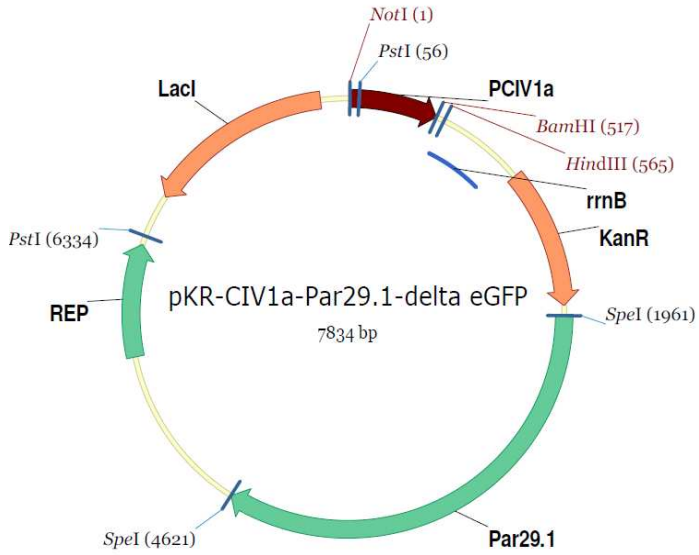


Figure 51: Vector map of pKR-CIV1a-Par29.1-ΔegfpΔlacI REP, containing the CIV1a promoter originating form R. eutropha H16, the REP origin of replication, the kanamycin resistance gene and the par29.1 sequence. egfp as reporter gene was deleted from this vector.

Appendix 3: Sequences

Terminator sequences from RK2 [30]:

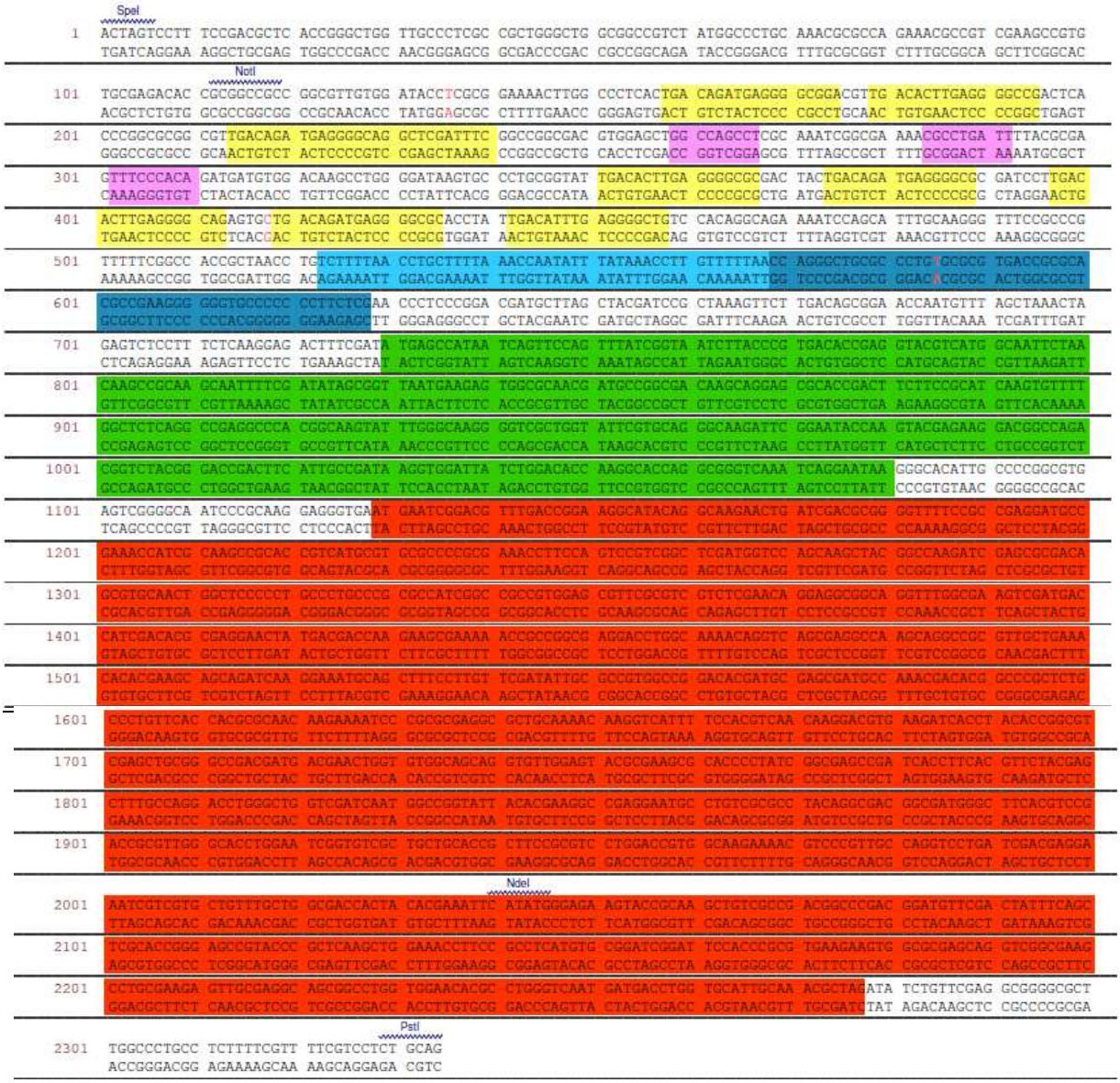
$T_{L13.2}$: ATATCTGTTCGAGGCGGGGCGCTTGGCCCTGCCTCTTTTCGTTTTCGTCCT

$T_{RL31.1}$: ATATCTGTTCGAGGCGGGGCGCTTGGCCCTGCCTCTTTTCGTTTTCGTCCT

Sequence of oriV393:

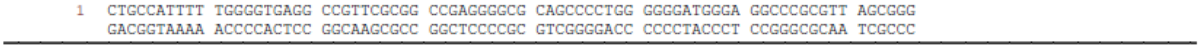
The DnaA binding boxes are marked in pink, iterons are marked in yellow, the AT-rich region in light blue, the GC-rich region in dark blue, the ssb sequence in green and the trfA gene in red. Mutations in the sequence (at 180 bp and at 347 bp) compared to the original sequence of the RK2 plasmid [30] are marked with red letters.

Sequence of oriV632:



The DnaA binding boxes are marked in pink, iterons are marked in yellow, the AT-rich region in light blue, the GC-rich region in dark blue, the ssb sequence in green and the trfA gene in red. Mutations in the sequence (at 180 bp and at 347 bp) compared to the original sequence of the RK2 plasmid [30] are marked with red letters.

Sequence of the pRK310 plasmid (derivate of pRK290): only the additional 76 bp are shown



Additional 76 bp of the oriV sequence of the pRK310 plasmid, unknown function;