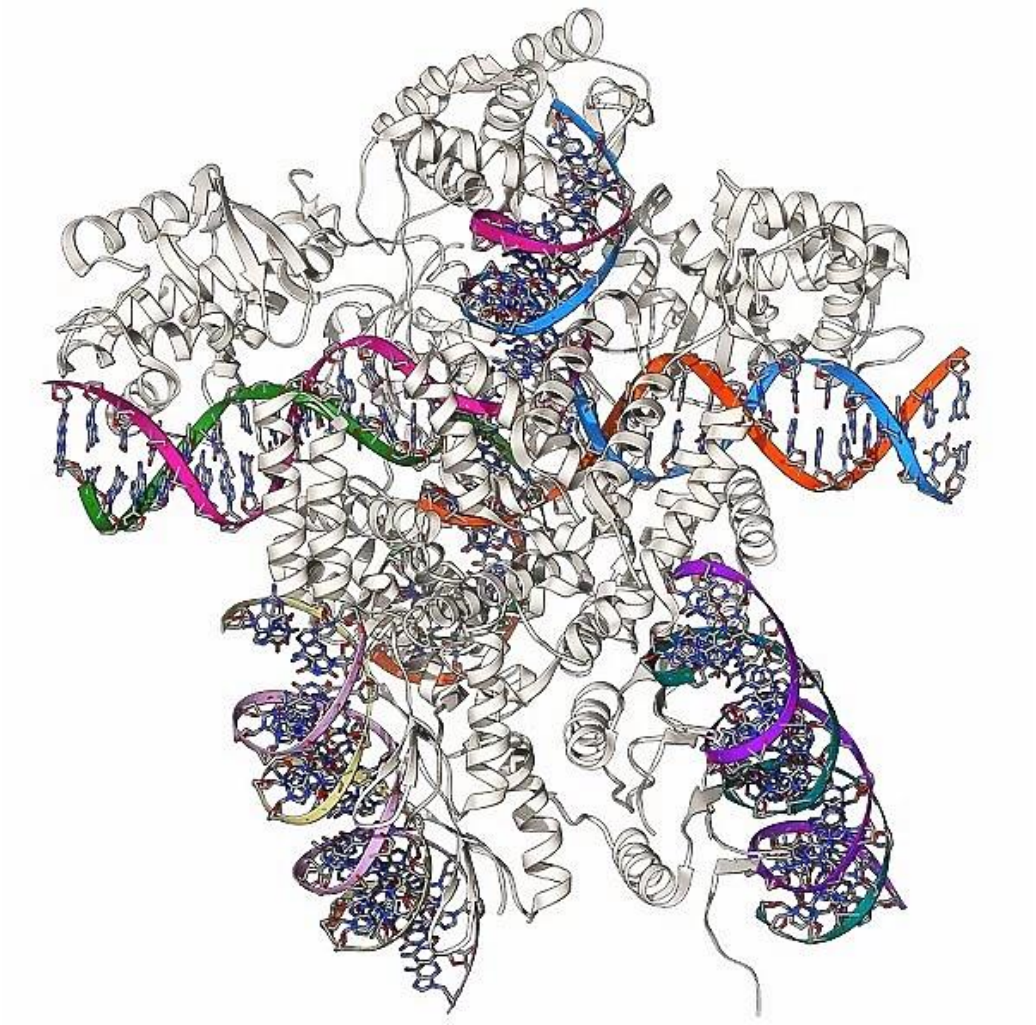
KU LEUVEN

FACULTY OF BIOSCIENCE ENGINEERING

Flip and rule

Exploring ways to test novel Pseudomonas bacteriophage-derived integrases



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Abstract

In synthetic biology (SynBio) there is a need for fast and reliable ways to build complex genetic circuits by inserting large stretches of DNA without relying on extensive sequence homology. Bacteriophage integrases can facilitate this. However, few integrases have been described for non-model organisms like *Pseudomonas aeruginosa*. This research discusses the development of test systems to validate integrases derived from a high-throughput genomic analysis of *P. aeruginosa*-specific bacteriophage genomes. Two general types of test systems were developed, with distinct outcomes: (1) insertion of the constitutive Pem7 promoter in front of the *msfgfp* marker gene, or (2) reversion of an inverted *msfgfp*. SEVAtile cloning was used to construct plasmids containing the integrase gene and corresponding *attachment sites* (att-sites) of the well-known integrases phiCTX (*P. aeruginosa*) and Bxb1 (*Mycobacterium smegmatis*). The integrases were tested under the control of either the rhaB-promoter or XylS/Pm inducible expression system. The first system involved three plasmids (integrase, attP, and attB). This system failed to demonstrate integration, partly due to integrase-associated toxicity. Indeed, toxicity assays revealed that the rhaB-promoter has reduced integrase-associated toxicity compared to the XylS/Pm expression system. The second system was inversion-based, with both att-sites on one plasmid. Since construction with SEVAtile proved ineffective, a step-wise development of this system is currently under development. This study demonstrates that a stringent, low copy-number plasmid is crucial for the control of integrase expression. Additionally, inversion is argued to be a more reliable output for an efficient integrase test system, because of the proximity of the att-sites. Once validated in *P. aeruginosa*, the system can be used for the high-throughput testing of novel integrases, expanding the SynBio toolkit for this non-model bacterium.

Keywords: integrases, *Pseudomonas aeruginosa*, SEVAtile, SynBio

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Introduction

Over the last decades, genetic engineering has become a key approach to gain a fundamental understanding of complex biological systems, and to construct cell-based methods for the production of relevant products, such as drugs or industrial compounds (Collins et al., 2010). However, engineering biological systems remains challenging due to nature's complexity and the lack of controllable tools, especially for non-model organisms. In the field of microbial synthetic biology (SynBio), the goal is to design and test controllable genetic circuits to study or influence certain cellular functions (Bowyer et al., 2020). A fundamental aspect to achieve stable circuits is the guided altering or introduction of specific DNA sequences into the genome of bacteria, archaea, or yeast. Examples include transposases and genome editing via clustered regularly interspaced short palindromic repeat (CRISPR)-associated nucleases such as Cas9 (Jinek et al., 2012). In this research, the focus lies on another widely applicable tool for site-specific insertion: integrases. These can be retrieved from the age-old nemesis of bacteria: the bacteriophage.

The most well-known aspect of bacteriophages is their ability to exert complete control over their host's metabolism to promote the large-scale production of new phage particles. This usually involves lysis of the bacterial cell and spread of bacteriophages to neighboring bacteria to repeat this lytic cycle. In contrast to strictly lytic phages, temperate bacteriophages can integrate and stably maintain their own phage genome into the host's genome. This generally occurs when conditions are less favorable for lytic infection, e.g. in smaller bacterial populations. In these situations, phage-encoded integrases act as DNA recombinases by inserting and excising the phage genome in a controlled, site-specific and unidirectional manner (Fogg et al., 2014; Thompson et al., 1987).

Integrases: mechanism and classification

Bacteriophage integrases can be part of two distinct recombinase families: serine- and tyrosine recombinases, named after the catalytic amino acid residue in the active site. Although they differ by protein structure and mechanism of action, the end result is the same and the overall recombination process includes many similar elements (Bowyer et al., 2020). In general, recombination occurs between two *attachment* (att) *sites* on the DNA, one on the bacterial genome (attB), and one on the phage genome (attP). The integrase associates with these sites and brings them together, followed by cleavage of the DNA backbones, which then become covalently linked to the integrase catalytic residue (Ser/Tyr). After the exchange of the double-stranded DNA segments, the end-result includes a stretch of DNA flanked by two hybrid attP-attB-sites termed attL (attB-attP) and attR (attP-attB) (**Figure 1**). In general, recombination is unidirectional, i.e. the hybrid att-sites do not recombine to form attB and attP again.

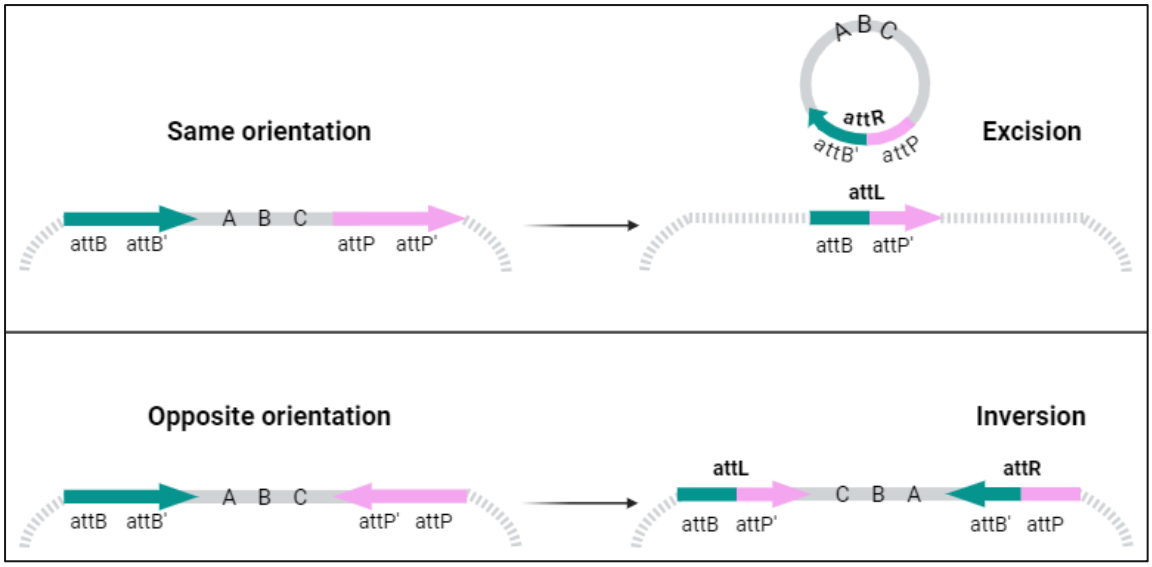

Figure 1. General concept of recombination

Two outcomes of integrase-mediated att-site recombination. The outcome is determined by the direction of the att-sites relative to one another. Although the reactions are unidirectional, as indicated by the arrows, the effects may be reversed under certain conditions with the help of an excisionase (see text).

Tyrosine recombinases are monomers that form a tetrameric complex around attB- and attP-sites, which are aligned antiparallel (**Figure 2-1**). Two Tyr-residues in opposite monomers become covalently attached to the 5'-ends of the DNA backbone on two sites (within attB and attP), while the 3'-ends become free. This is followed by transfer of these 5'-ends from the enzyme to the free 3'-ends of the opposite strands (Fogg et al., 2014). This produces a Holiday junction-like structure, which resolves after these steps are repeated for the other two monomers with the complementary strands. For effective Tyr-integrase action, the bacterial protein Integration Host Factor (IHF) sharply bends the DNA strands at the attP-site to allow correct formation of the synaptic complex. Within the family of Tyr-recombinases, a subdivision exists between unidirectional and bidirectional recombinases. While unidirectional Tyr-recombinases always require phage-encoded excisionase Xis along with host-encoded FIS protein, bidirectional Tyr-recombinases such as Cre and Flp can perform reversible DNA recombination cofactor-independently (Bowyer et al., 2020).

Well-known bidirectional Tyr-integrases include Flp, Cre and λ integrases, which have been widely applied in genomic engineering, e.g. in the removal of redundant (antibiotic) selection markers after stable integration of a plasmid (Hoang et al., 2000; Schweizer, 2003). Tyr-recombinases have also been used for the stable chromosomal integration of transgenes, for instance in the development of recombinant vaccines (Stover et al., 1991). An example of a unidirectional Tyr-integrase is the CTX integrase derived from phiCTX, a bacteriophage native to *Pseudomonas aeruginosa*. Although the use of this integrase is not as widespread in other bacteria, it is established as a tool in the *P. aeruginosa* research field (Qiu et al., 2006).

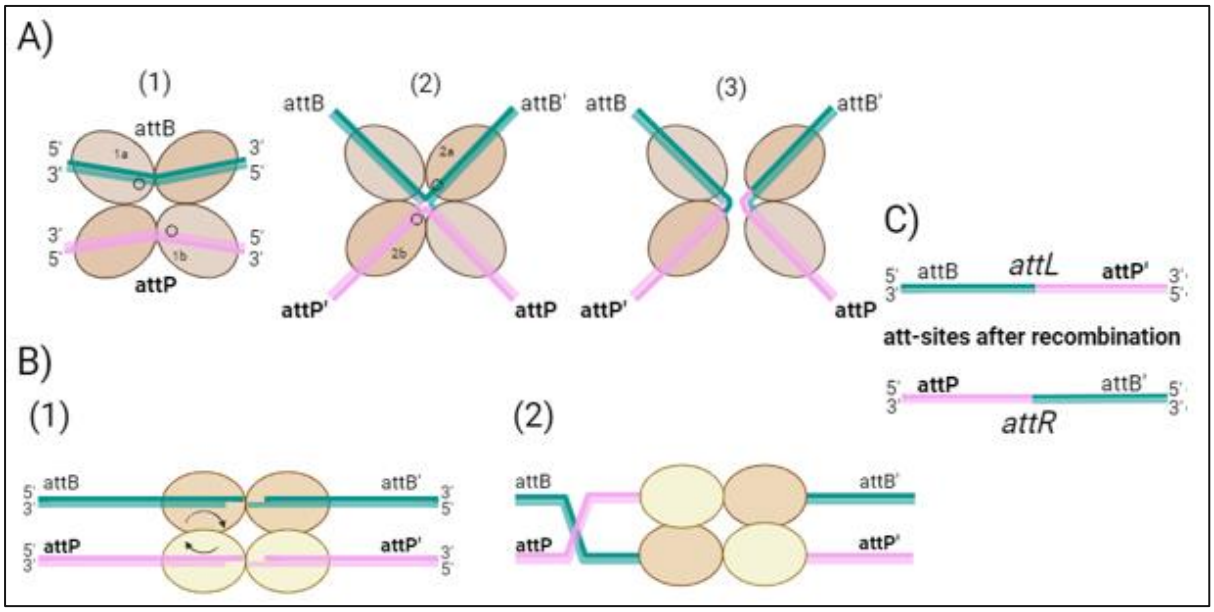

Figure 2. Mechanism of action of the two types of integrases
A) *Tyr-integrase mechanism. The attB- and attP-sites align antiparallel. Cleavage and ligation to the other strand occurs consecutively with (2) a Holiday junction as an intermediate. 1a-1b and 2a-2b are respectively the first and second set of Tyr-integrase monomers that cleave the DNA and attack the 5'-end with the crucial tyrosine residue.* ***B)*** *Ser-integrase mechanism. The attB- and attP-sites align in parallel. (1) Cleavage occurs simultaneously by all integrase-monomers and is followed by a 180° subunit rotation and (2) ligation to yield the recombined end-products.* ***C)*** *The att-sites after recombination. This end-result is the same for both types of integrases.*

The other family of recombinases are the serine recombinases. Although less common than Tyr-recombinases, they are equally interesting candidates for the SynBio toolbox. Ser-recombinases all act unidirectionally and are classified based on their size, which also dictates their structure and function. All Ser-recombinases have a catalytic domain at their N-terminus (the SR domain) and a C-terminal DNA-binding domain (Fan et al., 2016). In some Ser-recombinases, this domain contains a characteristic helix-turn-helix motif. These small enzymes are called DNA invertases or resolvases and catalyze inversions and deletions of small sections of DNA within the phage or bacterial genome (Johnson, 2015). They are typically around 180-200 amino acids long. Invertases and resolvases are structurally distinct from the group of large serine recombinases and have different mechanisms of action (Ghosh et al., 2005). While the small Ser-recombinases are involved in various other DNA

rearrangements within the phage genome, such as inversion or excision of specific DNA segments, large Ser-recombinases are the true 'Ser-integrases' that mediate integration and excision of phage genomes into and out of bacterial chromosomes.

The mechanism of Ser-integrases differs from that of Tyr-integrases. Ser-integrases first bind as dimers and then form a tetramer-like synaptic complex around two att-sites that are aligned in parallel (**Figure 2-2**). All four Ser-integrase subunits cleave the DNA backbones simultaneously, yielding two double-stranded breaks. This is followed by the removal of two nucleotides from the 3'-ends to create the dinucleotide overhang that is crucial in recombination polarity (Ghosh et al., 2005). Next, the free 5'-phosphates become covalently attached to the serines in the four subunits. This nucleoprotein synaptic complex is an intermediate structure that is unlike the Holiday junction created by Tyr-recombinases (Hirano et al., 2011). Next, two of the four subunits undergo a 180° subunit rotation, which repositions the associated double-stranded DNA next to the unrotated substrate DNA strands held by the opposite two Ser-integrase subunits. For Ser-integrases, the orientation of the att-sites has to be parallel for recombination to occur (Ghosh et al., 2005). An example of a Ser-integrase is the mycobacteriophage-derived Bxb1 int (*Mycobacterium smegmatis*), which has been a key player in the recent development of ORBIT, a high-throughput system to construct complex strains and mutant-libraries, also in non-mycobacteria such as *Escherichia coli* (*E. coli*) (Saunders & Ahmed, 2024).

Applications and advantages of bacteriophage integrases

Tyr-integrase CTX and Ser-integrase Bxb1 are just two examples of integrases that are currently used as SynBio engineering tools. Integrase research focuses on two main applications. First, the integrase specificity would allow for the controlled installation of novel genetic circuits at known/pre-determined sites in the bacterial genome, while preserving normal host metabolism. For example, in Recombinase-Mediated Cassette Exchange (RMCE), different stretches of sequence can be inserted into a 'landing platform' previously incorporated in the genome of the cellular or bacterial chassis. In *E. coli*, RMCE has been applied to delete genes and introduce large constructs (up to 10.3 kb) into the genome using the Ser-integrase phiC31 (Snoeck et al., 2019). Second, the unidirectionality of most integrases can be used for the creation of 'memory circuits' that register momentary environmental changes in a permanent way. An array of multiple orthogonally active integrases was constructed in *E. coli*, allowing for information-storage on a combination of 11 different conditions (Yang et al., 2014). Inversion of a marker gene after induction of an integrase reveals the presence of the inducer in a binary [0,1] format. Such information storage within bacteria could be applicable to permanently record certain environmental states/events in hard-to-reach places such as inside the human body, or to achieve sustained production of desired proteins in a bioreactor after only a one-time stimulus (Burrill & Silver, 2010).

Integrases demonstrate several advantages compared to the traditional toolbox of transposases and CRISPR-based engineering. Firstly, because integrases only require recognition of the two att-sites, no extensive DNA sequence-homology is required. This favors the integration of larger stretches of DNA, such as entire metabolic pathways or synthetic operons. Such long sequences would otherwise need to be flanked with long homologous sequences to achieve recombination (Snoeck et al., 2019). Secondly, with integrase-based methods there is no synthesis or degradation of DNA, which improves engineering accuracy. An additional advantage for Ser-integrases in particular is that they do not rely on endogenous repair pathways or host co-factors (like IHF) but that their action only requires the integrase and small att sites (Merrick et al., 2018). The main attractiveness of (Tyr-/Ser-)integrases for SynBio is in their ability to function completely orthogonally, i.e., without interfering with or dependency on any existing cellular process.

Reversibility

Even though the reaction of integrases is said to be unidirectional, there are ways to mediate excision. In nature, this only happens under suitable conditions (e.g., when the number of bacterial hosts in the environment is sufficient for the phages to propagate). In the lab, bacteriophage reactivation, which starts with excision, is induced by UV radiation, or by addition of antibacterial chemotherapeutic compounds (Landy, 1989). As discussed, unidirectional Tyr-integrases use the phage-encoded protein excisionase (Xis) and host-encoded protein FIS for this excision. Ser-integrases need only phage-encoded excisionase Recombination Directionality Factor (RDF). Identifying excisionases is more difficult than identifying integrases, because there is very little sequence similarity within this class of proteins (Hirano et al., 2011). Controlled excision would be useful in RMCE, e.g., to switch out gene A for gene B in the same location by recycling the same att-sites, instead of starting from scratch every

time or working with multiple concatenated att-sites belonging to different integrases, which may lead to interference if orthogonality cannot be ensured. Furthermore, although memory circuits initially depend on integrase directionality, it might be useful to 'reset' them and recycle the bacteria that contain these circuits for another sampling round. Thus, the conditional reversibility of the integration would add greatly to the potential of integrases in SynBio.

Application of integrases in non-model organisms

It is clear that bacteriophage integrases are promising tools for the genetic engineering of bacteria ('biological chassis'). In the selection of the most suitable bacterial chassis, it is important to consider the effects of the downstream industrial applications on the organism of choice. Since the start of the genomics-era of biology, *E. coli* has been the standard model organism for much of the SynBio work, since most molecular research focused on this bacterium. However, the production of complex synthetic compounds as well as bioremediation-like processes often yield intermediates that are toxic for *E. coli* (Nikel et al. 2014). For this reason, non-model bacteria have emerged as promising alternative SynBio chasses. One example is the *Pseudomonas* genus, which contains many different bacterial species. *P. putida* is an interesting candidate for environmental cleanup purposes, because it can survive in highly hydrophobic environments and is a good producer of polyhydroxyalkanoates, a sustainable alternative to oil-derived plastics (Nikel et al., 2014).

This article focuses on *P. aeruginosa*, an opportunistic pathogen that can cause severe infections in susceptible individuals, e.g., in hospitalized people, and most notably in cystic fibrosis patients (Ipoutcha et al., 2024). It has various virulence factors, conferring it with e.g. antibiotic resistance and the ability to form biofilms. Although these are unwanted characteristics in hospitals, they may be of use in SynBio, because they demonstrate the resilience of this bacterium in harsh environments. SynBio efforts to combat the pathogenicity of *P. aeruginosa* are also undertaken, e.g., by tailored modification of phages or the creation of next-generation vaccines by systematic removal of virulence factor encoding genes (Ipoutcha et al., 2024; Jinyong et al., 2024). Furthermore, the high number of *P. aeruginosa* isolates (from the infected sites) makes it a rich source to find various associated bacteriophages and integrases.

One of the challenges in moving *Pseudomonas* forward as a novel SynBio chassis is that the current toolkit for genetic programming, including integrases, is much more limited compared to *E. coli* and insufficient for the building of more complex circuitry. To expand the pool of candidate integrases, the first important step is to mine *Pseudomonas*-specific phages, since a phage's host-specificity confers an optimisation of phage protein function within their specific host. Earlier studies have undertaken a bioinformatics approach to uncover more phage integrases, especially large serine integrases that work as well as Bxb1 (Durrant et al., 2023; Yarnall et al., 2022). E.B. Gutiérrez performed a high throughput *in silico* analysis of 108 *P. aeruginosa* genomes and 490 associated phages, leading to the identification of 932 site-specific recombinases belonging to both *P. aeruginosa*-specific prophages (Gutiérrez et al., 2024). Eventually, a final set of 12 integrases was selected based on availability of the phages and a high sequence diversity in this selection. Additionally, the original att-sites of these new candidate prophages were determined by experimental phage infection of *P. aeruginosa* and prophage sequence identification (Gutiérrez et al., 2024).

The next step of this research is to test the efficiency of each candidate prophage. A 3-plasmid integrase efficiency test had been conceived, which would first need to be tested on already established integrases discussed earlier: the Tyr-integrase CTX, and the Ser-integrase Bxb1. This report describes the setting up of this system, the challenges in that process, and the design and setting up of different systems. Perspectives and other possibilities are discussed. The development of a robust and reliable test system is crucial for the next step in the quest for an expansion of the current collection of integrases to be used in the SynBio engineering of *P. aeruginosa*.

Materials & Methods

Bacterial strains, vectors

E. coli TOP10 was used to construct and amplify the pDes4 and pDes1 plasmids[1], as well as the mini-CTX2 and pSEVAtile Entry vectors. The mini-CTX2 vector was used as a template to retrieve the CTX integrase and its attP-site[2]. The pSEVAtile Entry vector was used to insert a new Pem7 tile (flanked by PM3a and PM2; subsequently used in pDes4). *E. coli* PIR2 was used for pBG13 plasmids[3] (ThermoFisher Scientific). The pSEVAtile Destination vectors (pDes4, pDes1, and pBG13) were consecutively transformed into the *P. aeruginosa* laboratory strain PAO1. Besides these PAO1 strains, an *E. coli* TOP10 strain was made containing a novel pSEVAtile Entry vector with Pem7 and the position markers 3a and 4.

[1](Lammens et al., 2022) [2](Hoang et al., 2000) [3](Zobel et al., 2015)

Table 1. List of PAO1 strains created in this work

*This is a comprehensive list of PAO1-strains used in this study. The name consists of the strain (PAO1), any inserted vector between colons, e.g. :pBG13-attP:, followed by any additional plasmid(s). All strains were made for both CTX and Bxb, indicated by 'CTX/Bxb1'. *Glycerol stocks made **The pDes1 original vector contained a small insert of 10 bp instead of the usual SacB gene.*

Strain name	**Insert – detailed**	**Usage**
PAO1:pBG13-attP_**CTX/Bxb1**:*	pBG13-attP-BCD2-msfGFP	Strain for transformation with **CTX or Bxb1** attB- or integrase-containing plasmids
PAO1:pBG13-attP:pDes4-attB_**CTX/Bxb1***	pBG13-attP-BCD2-msfGFP + pDes4-Pem7-attB	Template for transformation with pDes1-int of **CTX or Bxb1**
PAO1:pBG13-attP:pDes1-int_**CTX/Bxb1**	pBG13-attP-BCD2-msfGFP + pDes1-int	Integrase only (on pDes1), used for toxicity tests
PAO1:pBG13-attP:pDes4-int_**CTX/Bxb1***	pBG13_attP_BCD2_msfGFP + pDes4_int	Integrase only (on pDes4), used for toxicity tests
PAO1:pBG13-attP:pDes4-attB+pDes1-int_**CTX/Bxb1***	pBG13-attP-BCD2-msfGFP + pDes4-Pem7-attB + pDes1-int	Strain with all components, used for integrase tests
PAO1:pBG13-attP:pDes4-int+pDes1-attB_**CTX/Bxb1***	pBG13_attP_BCD2_msfGFP + pDes4_int + pDes1_Pem7_attB	Strain with all components, used for integrase tests
PAO1:pBG13:_**PC***	pBG13_original vector contains: Pem7-BCD2-msfGFP	Positive control in integrase tests: naturally has high GFP expression
PAO1_**NC1**	pBG13-attP-BCD2-msfGFP + pDes4-attB + pDes1 original vector**	Negative control in integrase tests: not expected to express GFP
PAO1_**NC4**	pBG13-attP-BCD2-msfGFP + pDes1-attB + pDes4 original vector	Negative control in integrase tests: not expected to express GFP

Growth media and antibiotic selection

The *E. coli* and *P. aeruginosa* strains were grown in sterile liquid Lysogeny Broth (LB: 10 g/L tryptone[1], 5 g/L yeast extract[1], 10 g/L NaCl[2]) or on solid LB-agar media (LB, 15 g/L Agar[1]) in petridishes with the appropriate antibiotics for each plasmid-bacterium combination (**Table 2**). For transformation in *E. coli* strains, Super Optimal broth with Catabolite repression (SOC) medium (20 g/L tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.2 g/L KCl[2], 0.95 g/L $MgCl_2$[3], 1.2 g/L $MgSO_4$[4], 3.6 g/L glucose[2]) was added after the heat shock to ensure optimal recovery. For integrase functionality tests that involved fluorescence measurements on the CLARIOstar (BMG Labtech), the bacteria were grown in minimal medium: either M9 (1x M9 minimal salts[5], 0.2% citrate[4], 2 mM $MgSO_4$[3], 0.1 mM $CaCl_2$[3]) or CAA medium (5 g/L acid hydrolysed casein/CAA[1], 1.18 g/L K_2HPO_4[3], 1 mM $MgSO_4.7H_2O$[3]) supplemented with 150 µM $FeCl_3$[3] before each use. Bacterial cultures (liquid/solid) were grown at 37 °C, agar plates were stored at 4 °C.

Glycerol stocks of new bacterial strains (Table X) were made in sterile 15% glycerol (150 µL 100% glycerol[6] mixed with 850 µL bacterial culture) and kept at –80 °C.
[1]Neogen, [2]Acros Organics, [3]Sigma [4]Sigma Aldrich, [5]BD Biosciences, [6]Thermo Scientific

Table 2. Antibiotic selection in* E. coli *and* P. aeruginosa *for the different plasmids
*There are three different SEVAtiledestination vectors and one entry vector. Three bacterial strains were used for either plasmid amplification (*E. coli*) or integrase testing (*P. aeruginosa*). Different (concentrations of) antibiotics were used, because of the multi-resistant profile of* P. aeruginosa*.*

Plasmid	Bacterium	Antibiotic	Concentration	Other components
pDes1	*E. coli* TOP10	Tetracycline (Tc)	15 µg/mL	5% sucrose
	P. aeruginosa PAO1	Tetracycline (Tc)	60 µg/mL	
pDes4	*E. coli* TOP10	Spectinomycin (S/Sp)	50 µg/mL	
	P. aeruginosa PAO1	Streptomycin (Sm)	200 µg/mL	2% glucose (only for pDes4-int)
pBG13	*E. coli* PIR2	Kanamycin (K)	50 µg/mL	
	P. aeruginosa PAO1	Gentamycin (Gm)	30 µg/mL	
pST_Entry	*E. coli* TOP10	Ampicillin (Amp)	100 µg/mL	

Primer design
In the amplification of all vectors and inserts, they were flanked with the corresponding position marker overhangs (PMs). Primers were designed to achieve this. Short primers consist of a DNA-binding part and an overhang that consists of the correct PM followed by a recognition site (RS) for a specific restriction enzyme. For the Bxb1 att-sites, primers encompassing the entire Bxb1 att-sites including PMs and RSs were made to make double-stranded DNA by annealing. Primer annealing was done by combining 10 µL from each 10 µM primer-dilution and incubating for five minutes at 95 °C, followed by gradual cooling off to room temperature inside the PCR machine or on the bench. For primer sequences see **Table S1**. All primers were ordered at Integrated DNA Technologies (IDT).

Polymerase Chain Reaction (PCR)
Three types of PCRs were performed, the reaction conditions for which depended on the type of polymerase. Each polymerase has a different level of accuracy/efficiency. Kapa and Phusion polymerases (Thermo Scientific) were used for vector- and insert amplifications, respectively. All PCRs were performed according to manufacturer's instructions. As PCR template, 10 pg/µL was used for pBG13, and 100 pg/µL for pDes4. DreamTaq polymerase (Thermo Scientific) was used for colony PCR to check for the presence of each fragment or to assess recombination after integrase induction. When obtaining and preserving bacteria for colony PCR, two methods were employed: (1) directly picking the colonies from the culture plate or (2) growing them in 100 mL LB with antibiotics in a flat bottom 96-wells plate and taking 2.5 µL per culture after 2 h of shaking incubation at 37 °C. The conditions for colony PCR differ between *E. coli* and *P. aeruginosa* (**Table 3**), but this was only applied for the last colony PCR (see **Figure 10**, page 19).

Annealing temperature and touch down PCR
For the PCR reactions of the three-plasmid system, the annealing temperature Ta was based on the Tm of the primer's DNA-binding part only (Ta=Tm-1). For all PCRs in the pDes4-int and the 5-in-1-plasmid system, the first 10 cycles were with annealing temperatures based on the Tm of the DNA-binding part only, and the next 20 cycles were done with annealing temperatures based on the Tm of the whole primer.

Table 3. Single colony PCR with DreamTaq DNA polymerase
*These are the conditions for colony PCR to check the sequence of the plasmid in colonies after transformation. *If the culture was derived from a liquid culture instead of a colony, 2.5 µL was taken and 18 µL or 18.25 µL of mQ added for* E. coli *and* P. aeruginosa*, respectively.*

	E. coli	*P. aeruginosa*
Culture (colony)*	0 µL	0 µL
Primer F (20 µM)	0.5 µL	0.5 µL
Primer R (20 µM)	0.5 µL	0.5 µL
DreamTaq polymerase	0.5 µL **0.5 U/µL**	0.25 µL **5 U/µL**
10x DreamTaq green buffer	2.5 µL	2.5 µL
dNTP mix (10 mM)	0.5 µL	0.5 µL
mQ*	**20.5 µL**	**20.75 µL**
Total	25 µL	25 µL

30 cycles

PCR conditions	
95 °C	5 min
95 °C	**30 s**
50 °C	**30 s**
72 °C	**1 min/kb**
72 °C	5 min
12 °C	pause

Gel electrophoresis

For the analysis of DNA lengths, PCR products were run on 1-1.5% agarose gels for fragments above 500 bp and 1.5% for fragments less than 500 bp in length. Gels were prepared by dissolving agarose (Nippon Genetics) via microwave heating in 1X TAE buffer (40 mM Tris-HCl (Sigma Aldrich) pH=7.2, 500 M sodium Acetate (NaOAc) and 50 mM Ethylenediaminetetraacetic acid (EDTA) (Acros Organics)). One drop of ethidium bromide (EtBr, Merck) was added per 50 mL gel. Samples were loaded with a 6X TriTrack loading dye (ThermoFisher Scientific). Samples in Dreamtaq green buffer were loaded directly. For large fragments, the Phage Lambda genomic DNA (PstI digested according to manufacturer's instructions, ThermoFisher Scientific) was used as a DNA ruler. For smaller fragments, the 100 bp DNA ladder was used (ThermoFisher Scientific). Gel electrophoresis was conducted at 250 V for 25 to 40 minutes.

DNA purification, gel extraction, and miniprep

For purification of PCR products, extraction of DNA from the agarose gel, and plasmid extraction from *E. coli* liquid cultures the GeneJet purification kit, GeneJet Gel extraction kit, and GeneJet plasmid miniprep kit (ThermoFisher Scientific) were used, respectively. The manufacturer's instructions were followed. To pellet the *E. coli* cells after overnight liquid culture, they were centrifuged for 10 minutes at 4,000 *g* in a Megafuge 1.0 (Heraeus). All purifications/extractions were followed by measurement of the DNA concentration on the SimpliNano (Biochrom) based on the absorbance at 260 nm. All DNA samples were stored at -20 °C.

DNA sequencing and analysis

New constructs were sequenced by Sanger sequencing with Eurofins GATC Biotech (Konstanz, Germany). Sequence results were analyzed using ContigExpress (component of Vector NTI Advancer suite 11.0) and later using Sequencher software (Sequencher™ 5.4.6- Build 46289).

Restriction reactions and separate ligations

In general, the restrictions were done in a 20 µL volume with an incubation of 1 to 2 hours at 37 °C, followed (if necessary) by a 20-minute heat-inactivation step to inactivate the restriction enzyme(s). The restriction enzymes AvrII[2], BamHI[1], BpiI[2], BsaI[1], Bsa-HFv2[2], EcoRV[1], HindIII[1], and PstI[1], were used according to the manufacturer's instructions. Separate restrictions were followed by ligation of the tiles in the vector for 16 h at 16 °C using 1 U/µL T4 ligase[1], according to manufacturer's instructions. In some cases, phosphatase treatment of restriction products was necessary prior to ligation to prevent religation of the insert or vector with the vector/insert that they were originally with. For this, FastAP was used according to manufacturer's instructions.

[1] Thermofisher [2] NEB

SEVAtile shuffling

In the SEVAtile system, each 'tile' to be inserted into the vector is flanked by PMs that are complementary to the PMs of the adjacent vector/tile. Restriction with one enzyme, e.g., BsaI or BpiI, exposes these 4-bp overhangs. Since there is only one restriction enzyme involved, restriction and ligation can be performed in one 'SEVAtile shuffling' reaction (**Table 4**).

Table 4. Shuffling reactions

The conditions for SEVAtile shuffling. This reaction can only be done when all tiles and the vector have the same restriction enzyme recognition sites.

Vector (100 ng/µL)	1 µL
Tile(s) (50 ng/µL)	1 µL
Restr.Enzyme (10 U/µL)	1 µL
T4 DNA ligase (1 U/µL)	3 µL
10x ligation buffer	2 µL
mQ	- (varies)
Total	20 µL

	Cycling conditions		
Restriction	**37 °C**	**2 min**	**30-50 cycles**
Ligation	**16 °C**	**3 min**	
Lig inactivation	50 °C	5 min	1 cycle
RE inactivation	65/80 °C	5 min	1 cycle
	12 °C	pause	

E. coli transformation

E. coli were chemically made competent for transformations via the Rubidium Chloride method (Hanahan, 1983). The competent bacteria were thawed on ice from a –80 aliquot, and 2-10 µL of the ligation mix was added, followed by a 30-minute incubation on ice. The bacteria were heat-shock treated for 30 sec at 42 °C, and immediately transferred to ice. After addition of 250 µL of SOC-medium without antibiotics, they were incubated shaking (~250 rpm) for 45 to 60 minutes at 37 °C. Each transformation was plated on LB-agar plates with antibiotics. The success of the transformations was assessed with colony PCR of picked colonies, as described above.

P. aeruginosa transformation

Transformations in *P. aeruginosa* were done via electroporation (Choi & Schweizer, 2006). Overnight liquid cultures were prepared in LB medium with antibiotics. The next day, the cultures were divided into two Eppendorf tubes per overnight culture and centrifuged for two minutes at 13,000 *g*. The medium was discarded, and the cells were washed 2-3 times in 1 mL 300 mM (=10%) sucrose. After centrifugation at 13,000 *g* for two minutes, pellets were resuspended in a total volume of 100 µL per transformation. Next, 300 ng of plasmid was added for integration or 1 ng for a transformation without integration. After a 5-minute incubation at room temperature, the bacteria were electroporated in 0.2 mm cuvettes (Bio-Rad) at 2.5 kV. Immediately after this, 900 µL LB medium was added and the bacteria were incubated shaking (~250 rpm) for 1.5 to 2 hours at 37 °C, before plating them on LB-agar plates with antibiotics.

Toxicity tests (spot-tests) – integrase induction on plates

For the spot-tests for the three-plasmid system, bacteria were grown overnight in liquid LB cultures. The next morning, 100-200 µL was transferred to fresh 4 mL LB with antibiotics. The tested PAO1:pBG13-attP were only transformed with the plasmid carrying the integrase gene. After the liquid cultures reached an OD_{600} of ~0.3, they were serially diluted tenfold (10^{-1} to 10^{-9}) in LB with the corresponding antibiotics. Next, 2 µL of each dilution was spotted on LB agar plates that contained the indicated concentrations of 3-methylbenzoate (3-mBz) or rhamnose (Rha) for systems with pDes1-int or pDes4-int respectively.

CLARIOstar experiments – integrase induction in culture

PAO1 strains were grown overnight in 4 mL liquid minimal medium (CAA or M9, see above). The next day, they were transferred to fresh minimal medium to exponential phase (final OD_{600} of ~0.3). They were induced in 200 µL in 96-well black microtiter plates with a flat transparent bottom (Greiner Bio-one) containing 0.5 mM of 3-mBz or 5 mM of Rha. Both culture turbidity (OD_{600} absorbance) and msfGFP fluorescence (excitation at λ 485-10 nm and emission at 528-10 nm, measured with enhanced dynamic range setting) were measured at 37 °C with a CLARIOstar multimode plate reader (BMG Labtech). The plate was shaken 30 s at 300 rpm before each cycle. The effect of integrase induction was studied up to 12 hours (1 measurement per 15 minutes, 48 cycles in total). Analysis was done in Microsoft Excel and fluorescence intensities were normalized to OD_{600}-values.

Results

In the past, different integrase test systems have been developed for use in *E. coli* and some other organisms (Durrant et al., 2023; Hoang et al., 2000; Snoeck et al., 2019). The aim of this research was to design and develop a test system to evaluate the efficacy of the candidate integrases discovered in the large-scale genome mining effort (Guitierrez et al. 2023) in *P. aeruginosa*. For a suitable system, the construction should be kept straightforward to enable testing with sufficient throughput. Additionally, the outcome of the test system should be measurable and unambiguous, which can be verified using known integrases CTX (Tyr-integrase) and Bxb1 (Ser-integrase). Since the outcome of recombination can differ based on the direction of the att-sites relative to each other (**Figure 1**), different systems to test integrase-efficiency can be devised, each with a different measurable outcome. In both systems, recombination leads to expression of the marker gene monomeric superfolder Green Fluorescent Protein (*msfgfp*). In the initial system, recombination would lead to insertion of a promoter upstream of the *msfgfp* gene. In the later systems, recombination reverses an inverted *msfgfp* gene to its correct reading frame, followed by msfGFP-expression.

The three-plasmid system

Design of the three-plasmid system

In the first system, the three principle components required for recombination (attP, attB, and the integrase) are each encoded on separate plasmids (Figure 3). The pBG13 plasmid contains the attP-site, followed by a bicistronic design ribosomal binding site (BCD2, Claassens et al., 2019) and the msfgfp marker gene. Since this pBG13-attP plasmid does not contain a promoter, there would normally not be any msfGFP-expression. The other two plasmids contain either the attB-site with a constitutive promoter (Pem7) in front, or the integrase gene (CTX or Bxb1) with an inducible promoter. All plasmids are 'destination vectors' from the SEVAtile system, which allows for the incorporation of multiple genes or regulatory elements as 'tiles' (Lammens et al., 2022). The integrase gene is present on pSEVAtileDes1 (pDes1) or pSEVAtileDes4 (pDes4), while the Pem7-attB is on the other vector (Figure 4). This allows for the testing of two types of induction systems: the XylS/P_m-promoter (on pDes1, inducible with 3-methylbenzoate), or the rhaB-promoter (on pDes4, inducible with rhamnose). The mechanism of this system is as follows: the pBG13-attP is incorporated into the PAO1 genome and the plasmids with the integrase and attB-sites are added later. After induction and expression of the integrase gene, recombination of the att-sites would bring the constitutive Pem7 promoter in front of the GFP gene, leading to measurable output in the form of GFP fluorescence (Figure 3).

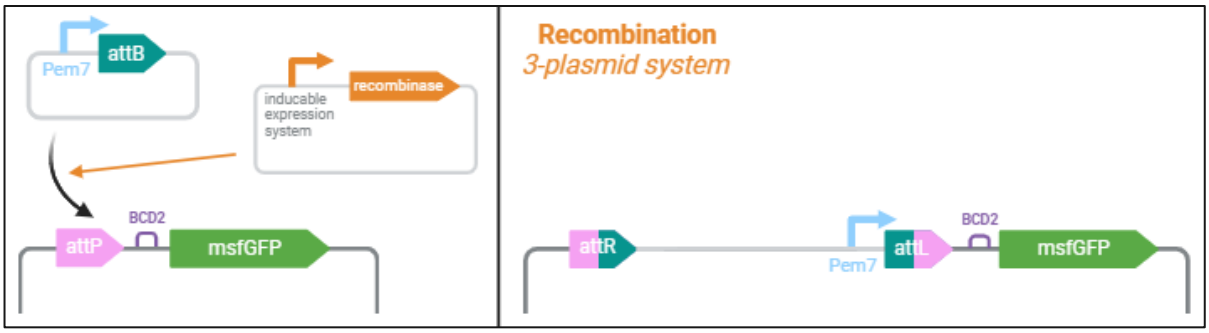

Figure 3. The outcome of recombination in the three-plasmid system
In this system, the attP-site is integrated into the PAO1 genome. Next, two other plasmids, with the attB-site located after a promoter (left) and with the integrase gene in an inducible expression system (right), are added. When these are both present and the integrase expression is induced, the genomic and plasmid-located att-sites can be recombined. In this case, recombination leads to integration of the entire attB-plasmid, positioning the Pem7-promoter just before BCD2-GFP and allowing constitutive expression of this marker gene.

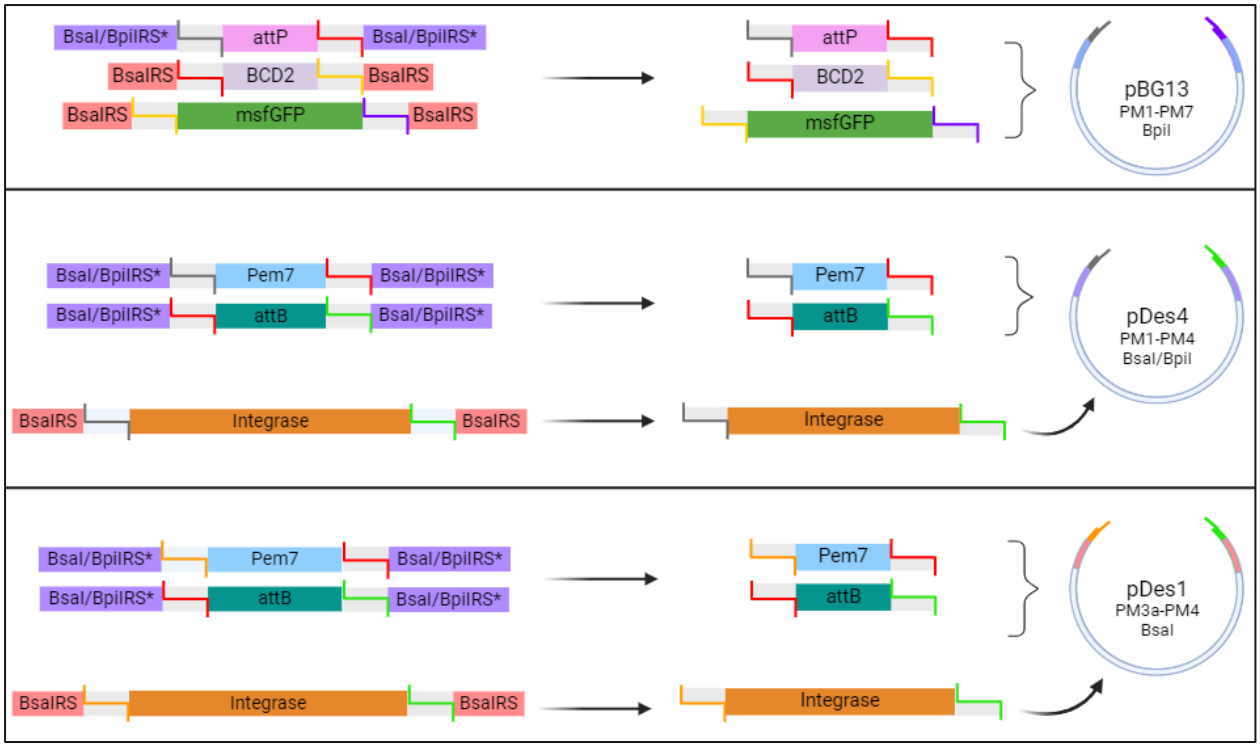

Figure 4. The SEVAtile cloning of plasmids for the three-plasmid test system
Graphic representation of the SEVAtile assembly of the plasmids for the three-plasmid system. The position markers of each tile are compatible with the vector and/or the adjacent tile(s). On the far end of each tile is a restriction site (RS) for BsaI (red) or BpiI (blue). Purple restriction sites indicate that these tiles are available with either the BsaIRS or the BpiIRS (BsaI for the CTX-tiles and BpiI for Bxb1-tiles). After restriction, 4-bp overhangs are created at each side of the tiles (and vectors). bp: base-pair, pDes1: PM: position marker, indicated by the light-grey boxes with the colored hooks. For the corresponding sequences, see ***Table S1****.*

Transformed PAO1 strains demonstrated poor bacterial growth in liquid cultures

The plasmids with their corresponding inserts were produced using SEVAtile cloning (**Figure 4**, see **Table S1** for the PM-sequences). This entails the concurrent restriction of the various "tiles" with the same Type IIs restriction enzyme, followed by ligation in one reaction. The choice for a specific restriction enzyme depended on the absence/presence of its recognition site (RS) in the sequence of the tile. For this reason, the pBG13-attP-BCD2-msfGFP_Bxb1 plasmid could not be made in one reaction due to a BsaI RS in attP_Bxb1 and a BpiI RS in msfGFP. After production and verification of the different constructs, they were transformed into the *P. aeruginosa* strain PAO1 by electroporation. First, pBG13-attP was electroporated together with the transposase plasmid pTNS2 to achieve integration of the attP-site into the PAO1-genome. This integration was verified with PCR (see also **Figure 10**, page 19). The next step was the transformation of the PAO1:pBG13-attP_CTX/Bxb1: clones with the int- and attB-plasmids. As simultaneous transformation of both plasmids proved inefficient, these transformations were mostly performed stepwise.

Even though the transformation plates contained bacterial colonies, when transferring these colonies to liquid overnight cultures in LB, especially in M9 and CAA media, they had remarkably poor growth rates or even showed signs of lysis. Multiple factors may have influenced this poor bacterial growth. Firstly, read-through of the conditional promoter may have led to premature expression of the integrase, which entails both a production burden and a risk for toxicity due to the enzyme's nuclease activity. This toxicity was assessed in the assays described in the next section. Additionally, the minimal media may have been more stressful for the bacteria as there was less growth in these media compared to standard LB medium. Finally, the combination of multiple antibiotics, required to maintain all plasmids, may have caused additional stress. On its own, antibiotics-related stress cannot be the only factor, as previous multi-plasmid transformations involving double and triple antibiotic selection had minimal effect on growth (Lammens et al., 2022). Moreover, tests using lower antibiotic concentrations (data not shown)

did not succeed to improve this poor growth. Nevertheless, the combination of all these factors may have resulted in the observed poor growth and lysis of the bacterial colonies under these experimental conditions.

Toxicity tests reveal CTX toxicity and the benefit of the rhamnose induction system

As the integrase was suspected to be the leading cause in the observed toxicity, its associated toxicity was tested. This was done by spotting small amounts of the bacterial culture in a series of tenfold dilutions on plates with different concentrations of the inducer. Induction was done with 3-methylbenzoate (3-mBz) for pDes1-int and rhamnose (Rha) for pDes4-int. The first spot-test with pDes1-int_CTX revealed considerable toxicity of this integrase, because the colony density decreased drastically with increased concentrations of 3-mBz (**Figure 5**), with no colonies at all for a 3-mBz concentration of 1 mM (**Figure S1**). Additionally, colony morphology looked less dense than the control group. Interestingly, Bxb1 appeared to be less toxic in this pDes1-system, with denser spots and the same decrease as the control group. This suggests that the observed toxicity is not simply due to a higher concentration of tetracycline but can be attributed to the integrase and its expression. Similar toxicity through leaky integrase expression has been observed in another study (Miyazaki & van der Meer, 2013).

An important rationale for testing the integrase in both pDes1 and pDes4 plasmids was the known property of pDes4 to possess a more stringent control of gene expression, i.e. it shows limited integrase expression in the absence of inducer. This is partly due to the addition of 2% glucose, which further inhibits the already stringent rhaB-promoter. Interestingly, for pDes4-int almost no toxicity was observed for either of the integrases, with better overall colony morphology (**Figure 6**). This suggests that the pDes4 vector is a more appropriate backbone to contain the integrase gene than the pDes1 vector.

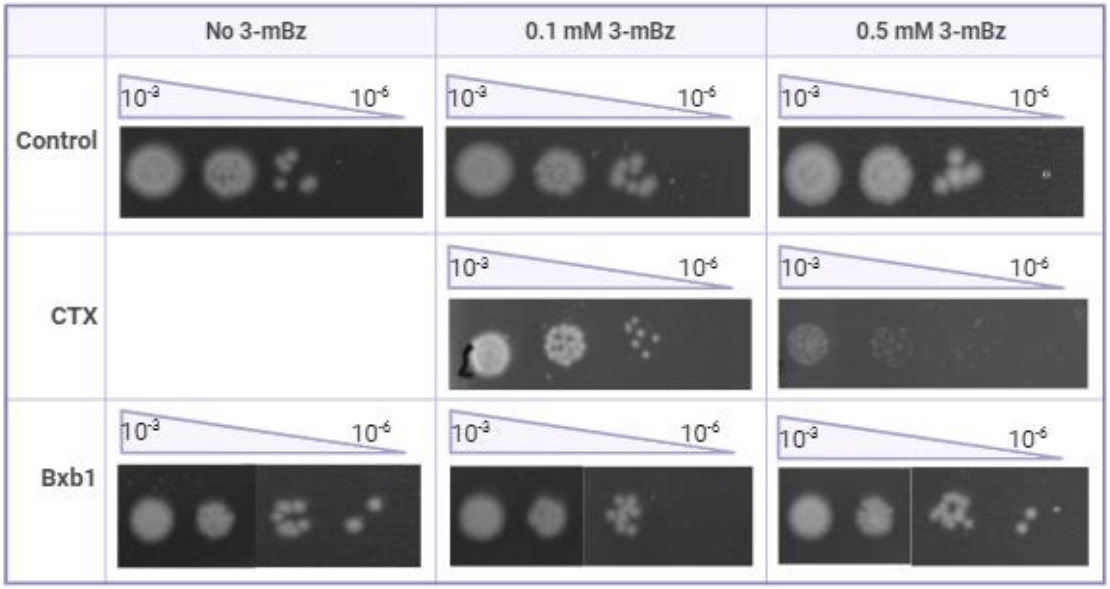

Figure 5. Toxicity of integrase expression in pDes1
The colonies of 'spots', serial tenfold dilutions from bacterial cultures that had reached an OD_{600} of ~0.3. The no 3-mBz group for CTX is not available. Control is PAO1:pBG13-attP:_Bxb1, CTX: PAO1:pBG13-attP:pDes4-attB+pDes1-int_CTX, Bxb1: PAO1:pBG13-attP:pDes4-attB+pDes1-int_Bxb1. 3-mBz: 3-methylbenzoate

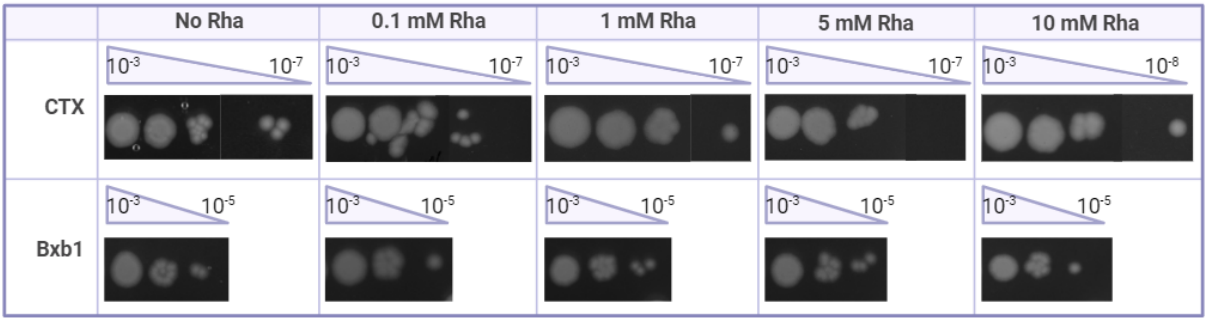

Figure 6. Toxicity of integrase expression in pDes4

The colonies of 'spots', serial dilutions from bacterial cultures that had reached an OD_{600} of ~0.3. Note that the 'No Rha'-plates also contained 2% glucose to restrict gene expression even more. CTX: PAO1:pBG13-attP:pDes4-int+pDes1-attB_CTX, Bxb1: PAO1:pBG13-attP:pDes4-int+pDes1-attB_Bxb1. Rha: rhamnose

The three-plasmid system does not demonstrate observable integrase activity after induction

After the completion of the stepwise transformation of PAO1, induction of integrase expression was performed in 96-wells plates while measuring msfGFP fluorescence with the CLARIOstar. Besides direct induction in the 96-wells plate on the day of the experiment, some of the tested strains were induced on LB agar plates first, to test another duration and mode of induction. After induction, the integrase-mediated recombination would definitively integrate the Pem7-promoter before the *msfgfp* gene, causing the fluorescence to rise over time (**Figure 4**). The positive control is a PAO1 wild-type transformed with the original pBG13 vector, which already contains Pem7-BCD2-msfGFP and thus constitutively expresses msfGFP. This positive control is expected to show the same (high) msfGFP expression level throughout the experiment, with or without induction. Negative controls were PAO1 with the attP-site that were transformed with the attB-plasmid and either the original pDes4 vector (instead of pDes4-int) or the pDes1 vector in which a short (10 bp) overlapping DNA sequence was inserted, replacing the SacB gene (instead of pDes1-int). On the CLARIOstar, these are expected to not display any msfGFP fluorescence, just like the uninduced test-samples.

When tested, none of the test samples (induced in 96-wells plate or on LB-plates) in either expression system showed a difference in msfGFP fluorescence, even after over 10h of induction (**Figure 7**). This was the case for both CTX and Bxb1. Despite the suboptimal bacterial growth observed in general, the experiments have been conducted using strains in the exponential phase (OD_{600} ~0.3) and growth curves were normal for all strains throughout the experiment (**Figure S2**). The GFP expression stayed the same for all clones throughout 12h of induction (**Figure S3**).

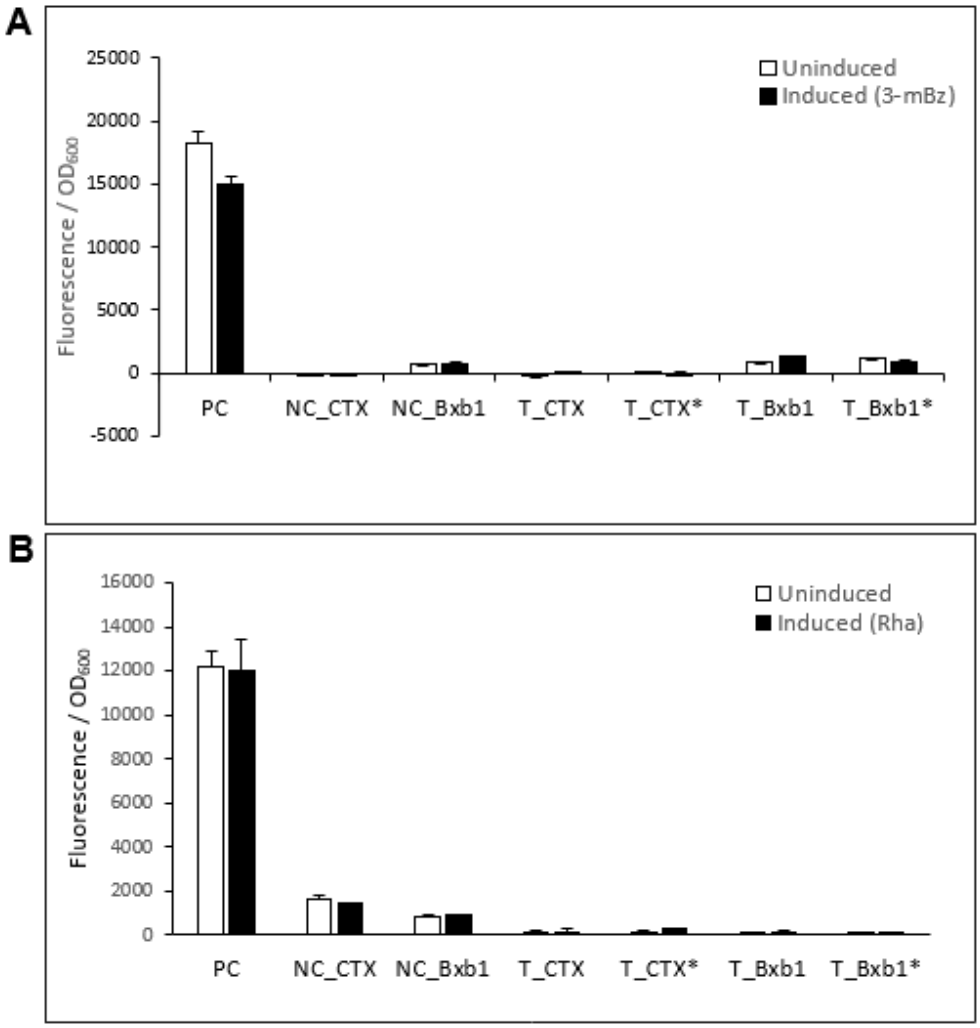

Figure 7. Fluorescence intensity after 10h induction with 3-mBz or Rha

*Graphs showing the fluorescence intensity at the GFP emission wavelength after ten hours, as recorded in the CLARIOstar (BMG Labtech). A) Induction with 3-mBz for the XylS/P_m expression system (with the integrase on pDes1), B) Induction with Rha for the rhaB-promoter expression system (with the integrase on pDes4). Experiments were conducted on different days, but all experimental conditions were identical (see Methods). PC: positive control, NC_CTX/Bxb1: negative control, these are different for the two different induction systems, T_CTX/Bxb1: the test strains that were transformed with all three plasmids (attP, int, and attB), *These test strains had previously been induced on plates. In the experiment, these strains were either incubated with or without an (extra dose of) inducer. All data was blank- and OD_{600}-corrected. Data and error bars represent the average and standard deviation for triplicates. All samples in the top graph, and NC_Bxb1 and plate-induced samples in the bottom graph represent n=3 technical replicates. The rest of the samples in the bottom graph represent n=3 biological replicates. 3-mBz: 3-methylbenzoate, Rha: rhamnose*

Although these results suggest recombination was unsuccessful, the lack of GFP fluorescence might be due to another factor. For example, the size of the attL site might influence the intensity of expression by increasing the distance between the Pem7 promoter and the *msfgfp* gene. To exclude such possibilities, separate tests were conducted to directly assess recombination at the DNA level. Colony PCRs were performed using two primer-sets: one for the integrated plasmid (pBG13, primer-set P), and one for the attB-plasmid (pDes4/pDes1, primer-sets B1/B4). Since the reverse primer is the same for both primer-sets, integration of the attB-plasmid would create new primer pairs and thus different lengths in PCR (**Figure 8**, **Table 5**). Primer-set P amplifies the attP-BCD2-GFP before integration (long fragment) and the attR after integration (short fragment). Primer-sets B1/B4 amplify the Pem7-attB on pDes1/4 before integration (short fragment) and the Pem7-attL-BCD2-GFP after integration (long fragment).

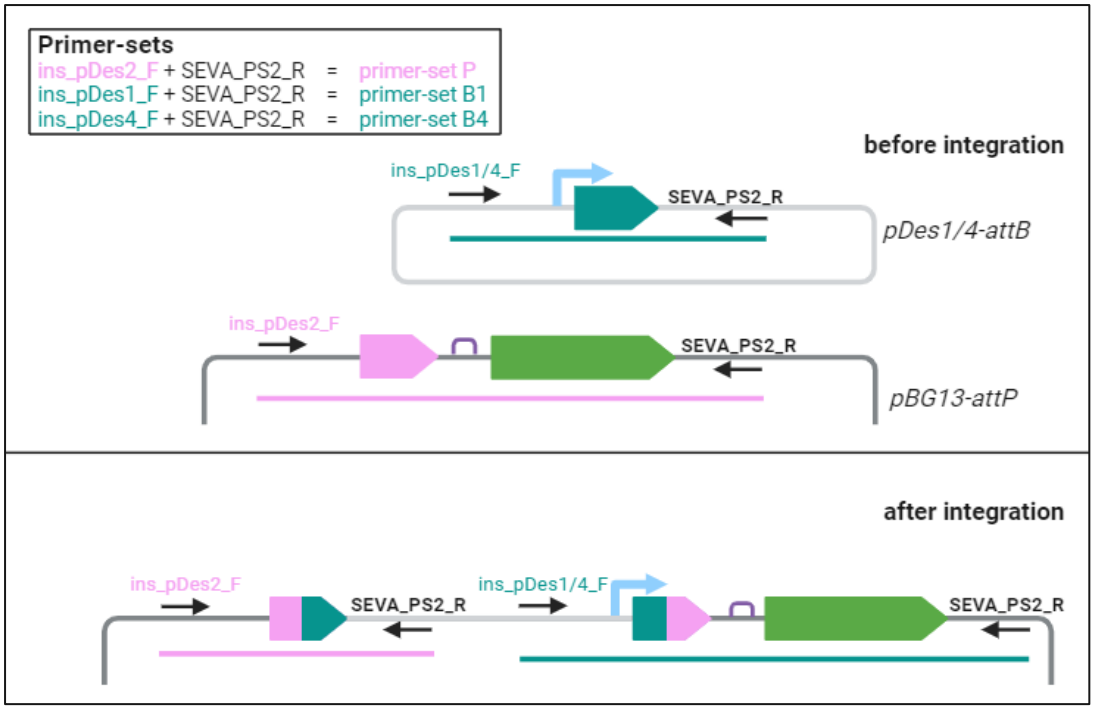

Figure 8. PCR check before and after integration

This figure shows the lengths of the sequences that will be amplified in PCR when using the SEVAtile primer-sets. The reverse primer is always the same (SEVA_PS2_R), but the forward primer differs. The pDes1/4_F and SEVA_PS2_R primers (dark cyan, ***primer-sets B1/B4****) will yield a short fragment before integration, covering the Pem7-attB segment. After integration, the pDes1/4_F primer would pair up with the SEVA-reverse primer on the integrated pBG13. This would yield a longer fragment after integration (Pem7-attL-BCD2-GFP). Meanwhile, the pDes2_F and SEVA_PS2_R primers (pink,* ***primer-set P****) will yield a long fragment before integration (attP-BCD2-GFP), and a shorter fragment after integration (only attR). ins: insert, pDes2: other name for pBG13*

As only one of these primer-sets is sufficient to demonstrate the occurrence of recombination, the B1/B4 primer-sets were used for triple-transformed PAO1 with CTX and Bxb1 integrases on the pDes4 vector – in which the least toxicity had been observed. Recombination was assessed for both uninduced, in LB-agar plate-induced and liquid culture-induced PAO1 (**Figure 9C**). The lengths expected for recombination were not observed, as all bands matched the size corresponding to a non-recombined system. This confirms the earlier results observed in the fluorescence experiments (**Figure 7**) and suggests that the three-plasmid system is not suitable as an integrase test system.

Table 5. Expected lengths with and without recombination

These are the expected lengths of the PCR products using the primer-sets shown in ***Figure 8****. The lengths of the CTX attL- and attR-sites were estimated by taking approximately half of the attB- and attP-sites. For Bxb1, the exact attL- and attR-site sequences have been described (Ghosh et al., 2005).*

	Primer-set	Recombination	No recombination
CTX system 1 (pDes4-attB)	P	~500 bp (pBG13-attR)	1467 bp (pBG13-attP-GFP)
	B4	~1600 bp (attL-GFP)	609 bp (pDes4-attB)
Bxb1 system 1 (pDes4-attB)	P	242 bp (pBG13-attR)	1157 bp (pBG13-attP-GFP)
	B4	1366 bp (attL-GFP)	447 bp (pDes4-attB)
CTX system 1 (pDes1-attB)	P	~500 bp (pBG13-attR)	1467 bp (pBG13-attP-GFP)
	B1	~1600 bp (attL-GFP)	562 bp (pDes1-attB)
Bxb1 system 1 (pDes1-attB)	P	232 bp (pBG13-attR)	1157 bp (pBG13-attP-GFP)
	B1	1328 bp (attL-GFP)	400 bp (pDes1-attB)

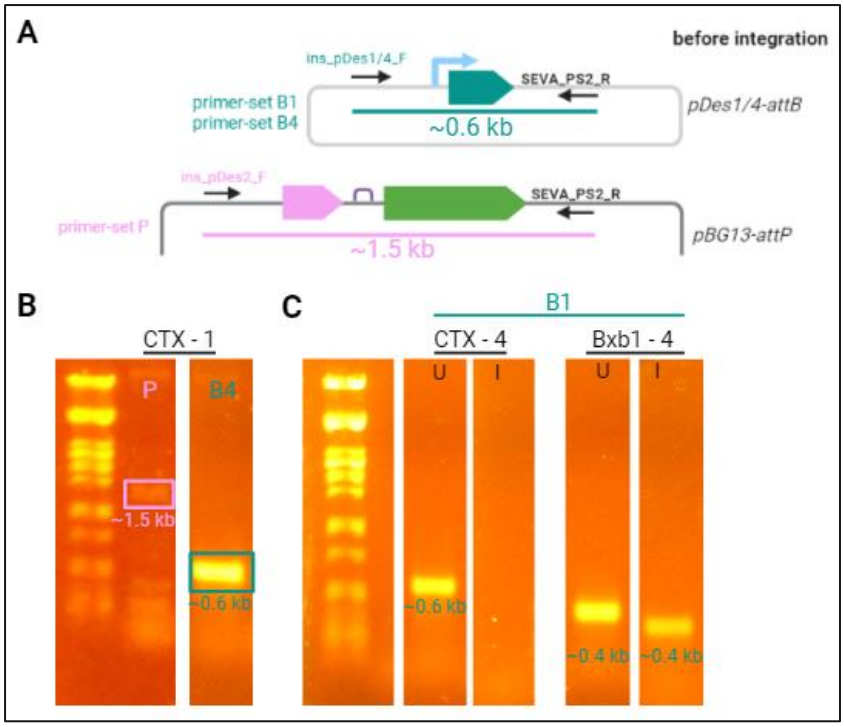

Figure 9. PCR of induced strains reveals that no recombination occurred

A) *To assess recombination, two primer-sets were used that would yield different lengths before and after integration. All observed lengths corresponded to the expected lengths before integration.* ***B)*** *As an illustration, both primer-sets were used for the induced PAO1 with pDes4-attB- and pDes1-int_CTX. Bands: 1467 bp for pBG13-attP-BCD2-GFP, 609 bp for pDes4-attB.* ***C)*** *For the pDes4-int test groups, only primer-set B1 was used. All observed lengths correspond to those expected before recombination. Bands: 562 bp for pDes1-attB_CTX, 400 bp for pDes1-attB_Bxb1. kb: kilobasepairs. U: uninduced, I: induced. Gels were snapped for clarity. For the full gel pictures, see Figure S4-S5. Both gels are 1% agarose gels with the phage lambda DNA PstI digest as a marker.*

Unexpected loss of the pBG13-attP insert in multiple test strains

PCR with primer-set B1 thus showed that no recombination **had occurred**. Before discontinuing the system, another assessment was done with primer-set P. Surprisingly, the final gel electrophoresis experiments only showed detectable bands for primer-set B1/B4, but not for primer-set P, with the forward primer on pBG13 (**Figure 9C**, **Figure S4**). This prompted another test to reassess the integration of the pBG13-attP plasmid at each step of the stepwise transformation process. Indeed, from this test it transpired that many test- and intermediate strains did not have the pBG13-attP site anymore (**Figure 10C**). This is surprising in light of the pBG13-attP integration checks performed in the past (**Figure 10A**). Furthermore, it does seem that something has integrated at the predetermined integration site (as confirmed with the primer-set GlmsDown and Tn7R, **Figure S6**). Perhaps a counterselection had arisen as a result of the alleged toxicity of the system that had given a selective advantage to bacteria that did not contain any plasmid (anymore). However, the recombination assessment of induced pDes1-int_CTX did show a band for the attP-or-attR-PCR (**Figure 9B**). Moreover, it is remarkable how despite the apparent loss of pBG13-attP in the first transformed strains or some of the intermediate strains, faint bands are visible for e.g. the triple-transformed pDes4-int_CTX transformation samples (**Figure 10C**). It would be preliminary to draw firm conclusions from these findings. Nevertheless, this data does give cause to question the reliability of the results obtained in the above-described induction experiment and is a reason for concern regarding the validity of the transformed PAO1 strains that have been established in this research.

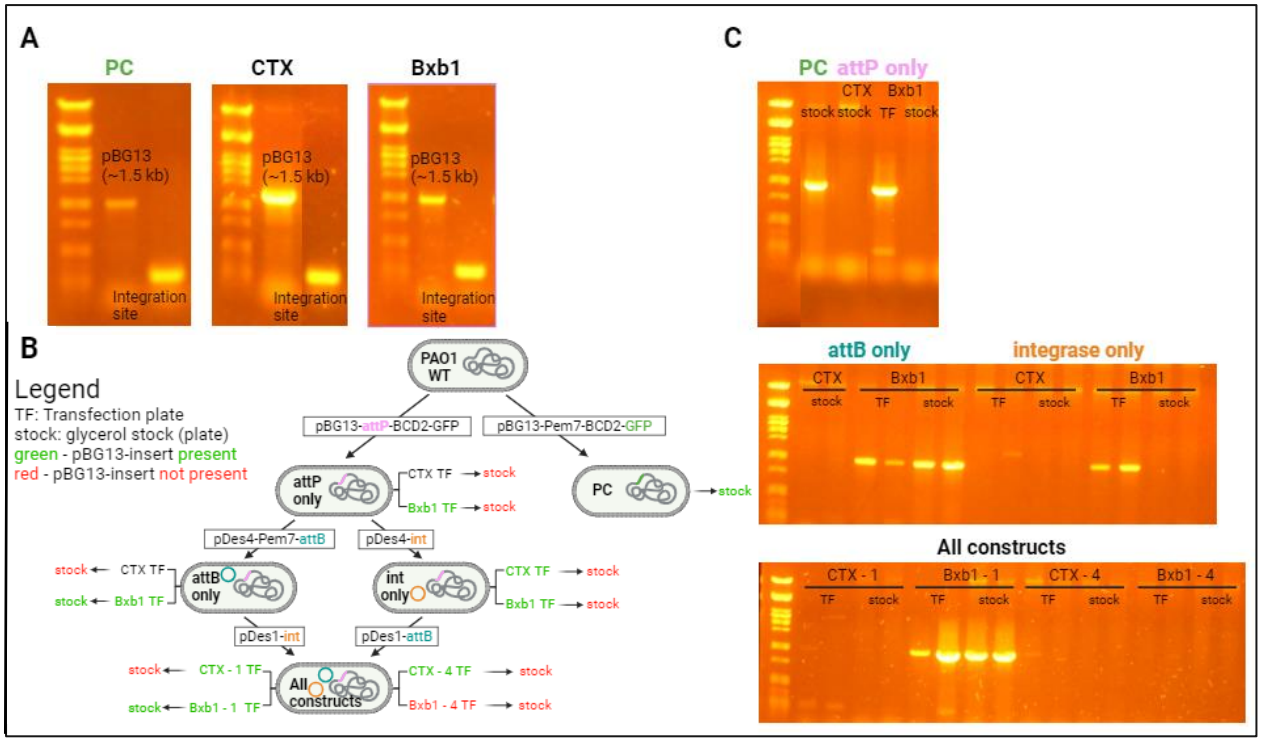

Figure 10. Repeated gel check to verify the presence of integrated pBG13-attP shows that it is missing in many of the intermediate clones

A) *First confirmation of pBG13-attP-BCD2-GFP integration into the PAO1 genome, performed after the first transformations in Pseudomonas. Both the positive control (PC) and the strains transformed with pBG13-attP_CTX/Bxb1 had successfully integrated the attP-site.* ***B)*** *This flowchart shows how the different strains were derived and interprets the gel images on the right. First, wildtype PAO1 (WT) is transformed with the normal pBG13 vector (forming the positive control, PC) or with the pBG13-attP_CTX/Bxb1 ('attP only'). The final strain with all plasmids is obtained in stepwise transformations, starting with the pDes4 vector with either the attB-site ('attB only'), or the integrase ('int only'), and followed by the pDes1 vector with the integrase or attB-site, respectively.* ***C)*** *Uninduced strains from all steps in the process. The first gel shows the PC and pBG13-attP-transformed strains that were also visible in A. The second and third gels contain the strains shown in the flowchart, loaded in duplicate. stock: colonies from plates on which the corresponding glycerol stock had been plated out, TF: colonies from plates derived from the original transformation plates. attP only: PAO1:pBG13-attP:CTX/Bxb1, attB only: PAO1:pBG13-attP:pDes4-attB_CTX/Bxb1, int only: PAO1:pBG13-attP:pDes4-int_CTX/Bxb1. All constructs: PAO1 transformed with all three plasmids, indicated as CTX/Bxb1 - 1 for pDes1-int and CTX/Bxb1 - 4 for pDes4-int. All gels contain the PCR products of the primer-set P (Figure 8) from colonies on LB agar plates. All gels are 1% agarose gels with the phage lambda DNA PstI digest as a marker.*

Prospects of the three-plasmid system

It is evident that with these two variations of the same type of test system it has been challenging to establish a reliable system that could be used to test novel candidate integrases. Although there may still be room for troubleshooting and adjusting the current state of this system, the consistently poor bacterial growth and the apparent loss of pBG13-attP in most glycerol stocks truly provoke a reconsideration of the general concept of the desired integrase test system and a critical evaluation of its design. One theoretical concern here is whether the integrase can effectively locate and unite the plasmid-borne attB-site with the genomic attP-site. More technical hurdles are the challenges in the consecutive cloning and triple transformation of *P. aeruginosa*, as discussed above. The triple transformations may pose a significant burden on the bacteria through integrase toxicity, selection with multiple antibiotics, and the use of low-nutritious minimal media. To circumvent these issues, the rest of this report will address the development of another type of test system in which the attB- and attP-sites are no longer on separate plasmids.

The 5-in-1-plasmid system

Design

To design a new system in which the amount of plasmids is reduced to solve the technical issue of toxicity and the practical issue of spatial att-site separation, the attP- and attB-sites will both be present on the same plasmid (pBG13). This time, the outcome of recombination will be the inversion of the DNA segment located between the two att-sites, which are positioned in the opposite orientation towards each other (**Figure 11**). In this set-up, the new pBG13-construct would consist of five consecutive SEVAtiles: Pem7, attB, BCD2-GFP (in the reverse orientation), and the attP-sequence. Since the direction of the att-sites is not certain, two orientations of the attP-site were tested, termed attP ("forward") and Ptta ("reverse"). The second plasmid, containing the integrase under the influence of the rhaB expression system, has already been made in the previous section (pDes4-int_CTX/Bxb1).

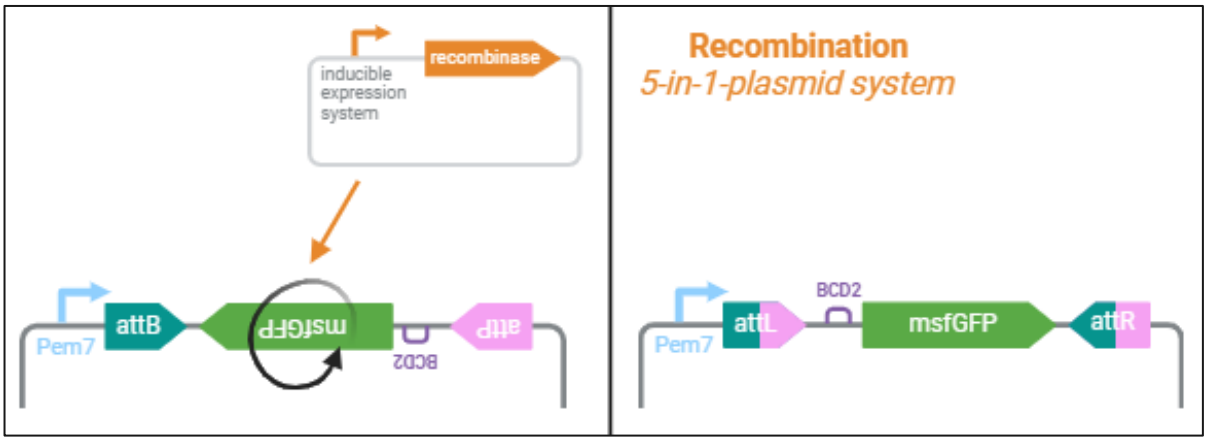

Figure 11. The outcome of recombination in the 5-in-1-plasmid system
In this system, the entire 5-tile construct is integrated into the PAO1 genome. Next, a plasmid with the integrase gene in an inducible expression system is added (pDes4 with the rhaB-promoter, from the three-plasmid system). The integrase catalyzes the recombination of the att-sites, which leads to the inversion of the previously inverted BCD2-msfGFP segment. This repositions the GFP-gene in the correct direction and, under the influence of the Pem7 promoter, constitutive expression follows as a measurable output of recombination efficiency.

Challenges with the implementation of the 5-in-1-plasmid system

For the creation of this 5-tile-plasmid, it was necessary to produce some of the tiles with new position markers (PMs) to connect them to one another (**Figure 12**). This illustrates the advantages of SEVAtile, i.e., that the same cloning strategy could be used, allowing for recycling of some of the tiles from the previous system. The tiles were successfully constructed (**Figure 13**). Restriction and ligation of the tiles to each other and into pBG13 was approached in different ways. First, all tiles were restricted separately and ligated into pBG13. Second, the tiles with the same recognition sites were restricted and ligated together in a SEVAtile shuffling reaction. Such 4-tile shuffling reaction (of Pem7, attB, inverted-msfGFP, and inverted-BCD2) could be done for CTX only, because both CTX att-tiles are restricted with BsaI, just as the inverted BCD2- and GFP-tiles. The 4-tile shuffling reaction was then followed by a ligation reaction of the 4-tile product, the attP-site, and the pBG13-vector, previously restricted with BpiI. After transformation of the ligation products into *E. coli*, the sequence lengths of the plasmids were verified in colony PCR (**Figure S7**). Most colonies showed lower bands than expected (1154 bp), indicating that the restriction-ligation reactions had only partly succeeded. This was confirmed by sequence analysis, which revealed that most of the times only one of the fragments was inserted, but never all of them (**Table 6**, **Figure S8**).

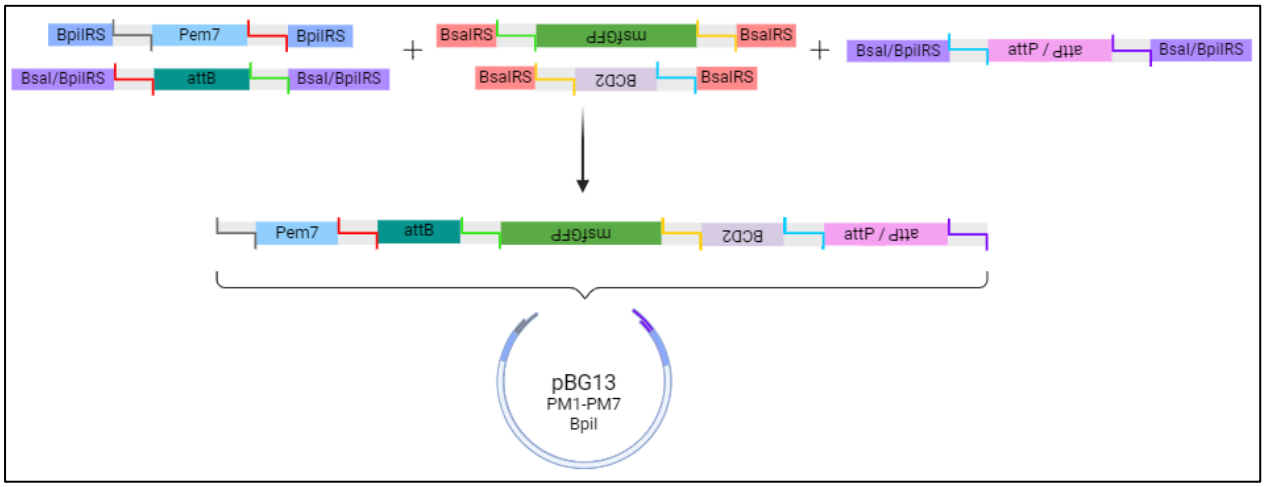

Figure 12. The SEVAtile cloning of plasmids for the 5-in-1-plasmid test system

Graphic representation of the SEVAtile assembly of the plasmids for the 5-in-1-plasmid system. The position markers of each tile are compatible with the vector and/or the adjacent tile(s). On the far end of each tile is a restriction site (RS) for BsaI (red) or BpiI (blue). Purple restriction sites indicate that these tiles are available with either the BsaIRS or the BpiIRS (BsaI for the CTX-tiles and BpiI for Bxb1-tiles). After restriction, 4-bp overhangs are created at each side of the tiles (and vectors). bp: base-pair, PM: position marker, indicated by the light-grey boxes with the colored hooks. For the corresponding sequences, see ***Table S1****.*

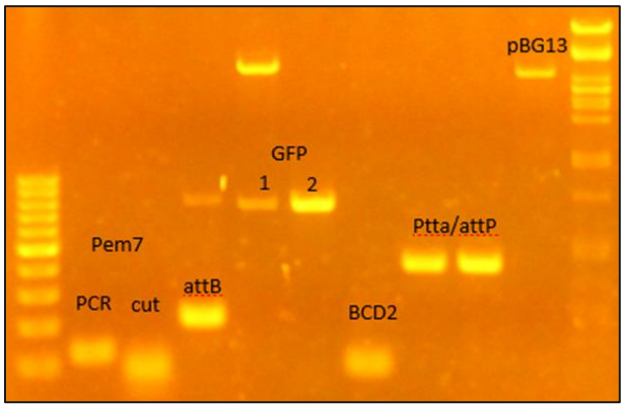

Figure 13. Gel displaying the restriction products of the five tiles and pBG13

The different tiles were successfully restricted after PCR amplification (Pem7, GFP-2, BCD2, Ptta/attP, and pBG13) or cut from the vector (attB from pDes4, GFP-1 from SEVAtile vector). For Pem7, the PCR product was also put on the gel (left). The attB, Ptta, and attP-sites on this gel belong to CTX. For Bxb1, primer-annealed products already had the correct PM-overhangs and therefore did not need to be restricted. This is a 1% agarose gel with the 100bp ladder (left) and the phage lambda DNA PstI digest marker (right).

Table 6. Overview of the sequencing results of the 5-in-1-plasmid system

The assembly of five tiles into one plasmid proved to be more challenging than anticipated. Most assemblies could not even be set up, with only the forward or reverse reads mapping onto the pBG13 vector. Analysis: contigExpress (Vector NTI Advancer 11). attP: the construct would contain the attP-site in the commonly annotated form, Ptta: the construct would contain the attP-site in the reverse direction to the commonly annotated one.

	Sequence results
CTX – attP	1) Empty vector 2) No assembly made
CTX – Ptta	1) No assembly made 2) No assembly made
Bxb1 – attP	1) No assembly made 2) No assembly made
Bxb1 – Ptta	1) Only Pem7 and reversed BCD2-GFP 2) Only reversed BCD2-GFP

Prospects of the 5-in-1-plasmid system

It appears that the bottleneck in the production of this 5-tile-plasmid is in the ligation of all tiles combined. It proved exceedingly challenging to strike the correct balance of molar concentrations used in such a massive ligation reaction, despite the fact that SEVAtile cloning has habitually been compatible with up to seven tiles (Lammens et al., 2022). The short length of many of the tiles undoubtedly plays a role in this: 4/5 Bxb1 tiles were smaller than 100 bp, and 4/5 CTX tiles were smaller than 400 bp. Furthermore, the ligation reaction had already undergone some troubleshooting before transformation even succeeded (data not shown). Finally, it was decided to stop the work on this system, for such a complicated cloning procedure will not be amenable for a high-throughput testing system for new integrases. Nevertheless, the general concept of (re-)inverting the marker gene through recombination remained appealing. The main objective for the next variation of this system would thus be to try a more 'fail-safe', stepwise approach.

The modified pBG13 vector

Design

As the intended SEVAtile-based shuffling approach could not be realized as intended, another method to make the desired 5-in-1 construct is necessary, preserving the pBG13 vector as the backbone (**Figure 16**). Although the outcome of this system is the same as the 5-in-1-plasmid system (**Figure 11**), the construction will proceed in a more stepwise fashion with the aim to ease the whole process. Starting from the original pBG13-vector, which already contains an *msfgfp* gene under the control of the constitutive Pem7 promoter, the BCD2-GFP segment would be inverted by PCR, restrictions, and ligation, and later flanked by the integrase-specific att-sites (**Figure 14**). In this way, the new 'starting point' of the test system is moved further upstream in the cloning process. Thus, for each novel integrase to be tested, one could start from this plasmid with the reversed marker gene to clone only the two att-sites, without the need to ligate all five tiles simultaneously.

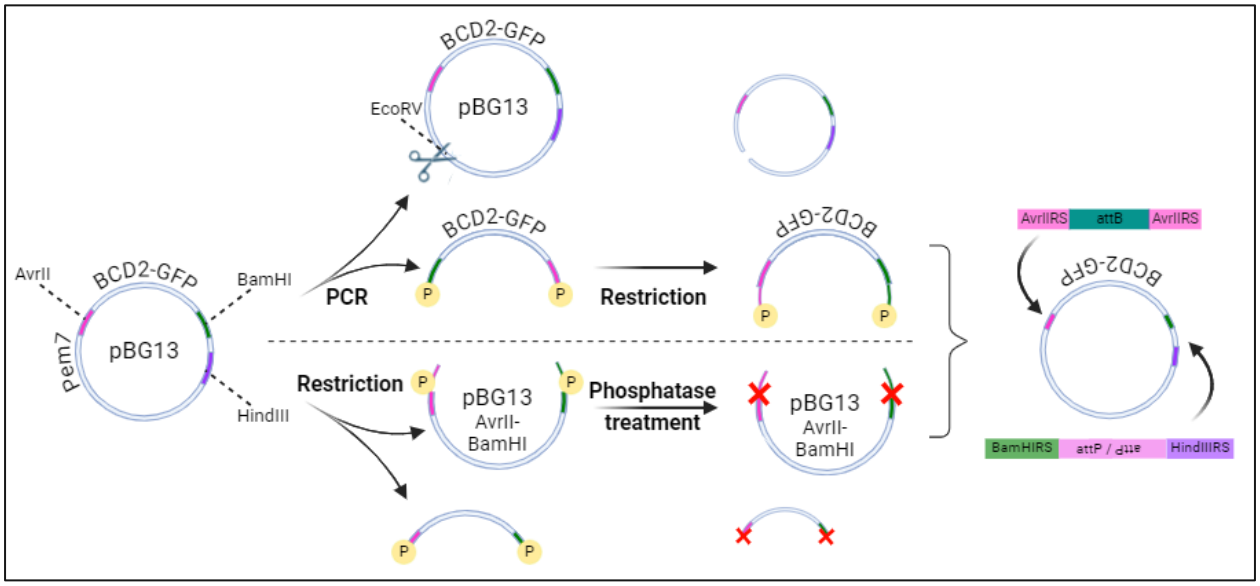

Figure 14. The concept of pBG13 vector modification
To create a modified pBG13 plasmid in which the BCD2-GFP segment is inverted, the original pBG13 vector was modified in two ways: a PCR reaction was performed to retrieve the BCD2-GFP segment with switched restriction sites. Restriction of the PCR product (top) and another sample of original pBG13 (bottom) created overhangs specific for the AvrII and BamHI restriciton sites. To exclude recircularization of the original pBG13 vector, the PCR mix (top) was treated with EcoRV to cut open the original vector, and the vector restriction mix (bottom) was treated with FastAP phosphatase, allowing only the 'inverted' BamHI-BCD2-GFP-AvrII fragment from the PCR mix to ligate into the AvrII-pBG13-BamHI open vector from the restriction mix. Once the modified pBG13 is created, the integrase-specific att-sites can be inserted at the 3' site (with AvrII) or at the 5' site (with BamHI and HindIII). P: phosphate-group at the free 5'-end, RS: Restriction Site.

Cloning of the modified pBG13 vector has been successful thus far

First, primers were designed to PCR the BCD2-GFP segment out of the vector (**Table S6**). The result is the following insert: BamHI-BCD2-GFP-AvrII (abbreviated as B-BG-A), which was confirmed on a gel (**Figure 15**). Next, both pBG13 and the B-BG-A fragment were restricted in a stepwise fashion with BamHI and AvrII. To prevent re-ligation of the removed insert into the original vector, the restriction product of pBG13 was phosphatase-treated and the PCR-mix containing B-BG-A was restricted with restriction enzyme EcoRV to break up any residual pBG13-vector.

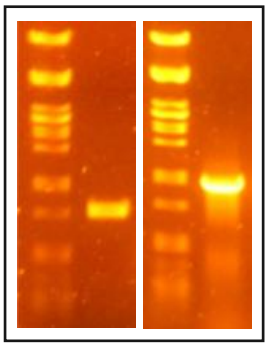

Figure 15. B-BG-A insert and colony PCR check
Left: This is the result of the PCR reaction to amplify the BCD2-GFP fragment and provide it with the BamHI- and AvrII-restriction sites at the BCD2- and GFP-ends respectively. Right: modified pBG13 after transformation into E. coli *PIR2. Both gels are 1% agarose gels with the phage lambda DNA PstI digest as a marker.*

Transformation of the ligated pBG13-B-BG-A ('partly modified pBG13') proved successful, as confirmed by both PCR and sequencing (**Figures 15**, **S9-S10**). Remarkably, however, the pellets of four sampled clones were all green, suggesting msfGFP-fluorescence, which should not be possible for inverted msfGFP (**Figure S11**). This seemed to contradict the results of the Sanger sequencing, which had revealed that the BCD2-msfGFP sequence was truly inversed (**Figure S10**). This unexpected finding may be explained by the observation that the T1 transcriptional terminator, located upstream from the gentamycin resistance gene (**Figure 16**), does not block transcription with a 100% efficiency (Putzeys et al., 2024). Nonetheless, if the final modified pBG13 vector is inserted in the PAO1 genome, this read-through will no longer be possible, because in the then linearized version of the plasmid, the resistance gene will point outward, away from BCD2-msfGFP.

Prospects of the modified pBG13 vector

At the moment, the att-sites with the correct overhangs have been produced and are ready to be inserted into the modified pBG13 plasmid (**Figure S12**). The next steps of this system have not been performed yet. First, the modified pBG13 should be restricted with AvrII, followed by ligation of the already restricted CTX/Bxb1 attB. Afterwards, another double restriction with BamHI and HindIII would prepare the attB-containing modified pBG13 vector to receive the corresponding B-Ptta/attP-H as an insert. Optionally, there could be an intermediate sequence check after incorporation of the attB-site. Moreover, given the unexpected green fluorescence in *E. coli*, baseline fluorescence in *P. aeruginosa* needs to be assessed. Even though this "final" system is still in development, several preliminary comments can be made. The main objective for a suitable test system is that it can be scaled-up for the high throughput testing of multiple integrases. Compared to the 5-in-1-plasmid system, the modified pBG13 vector clearly has more potential for success. However, since all integrases have their own attB- and attP-sites, the last steps will need to be repeated for each new integrase. The potential of this system will therefore depend on the ease of its future development.

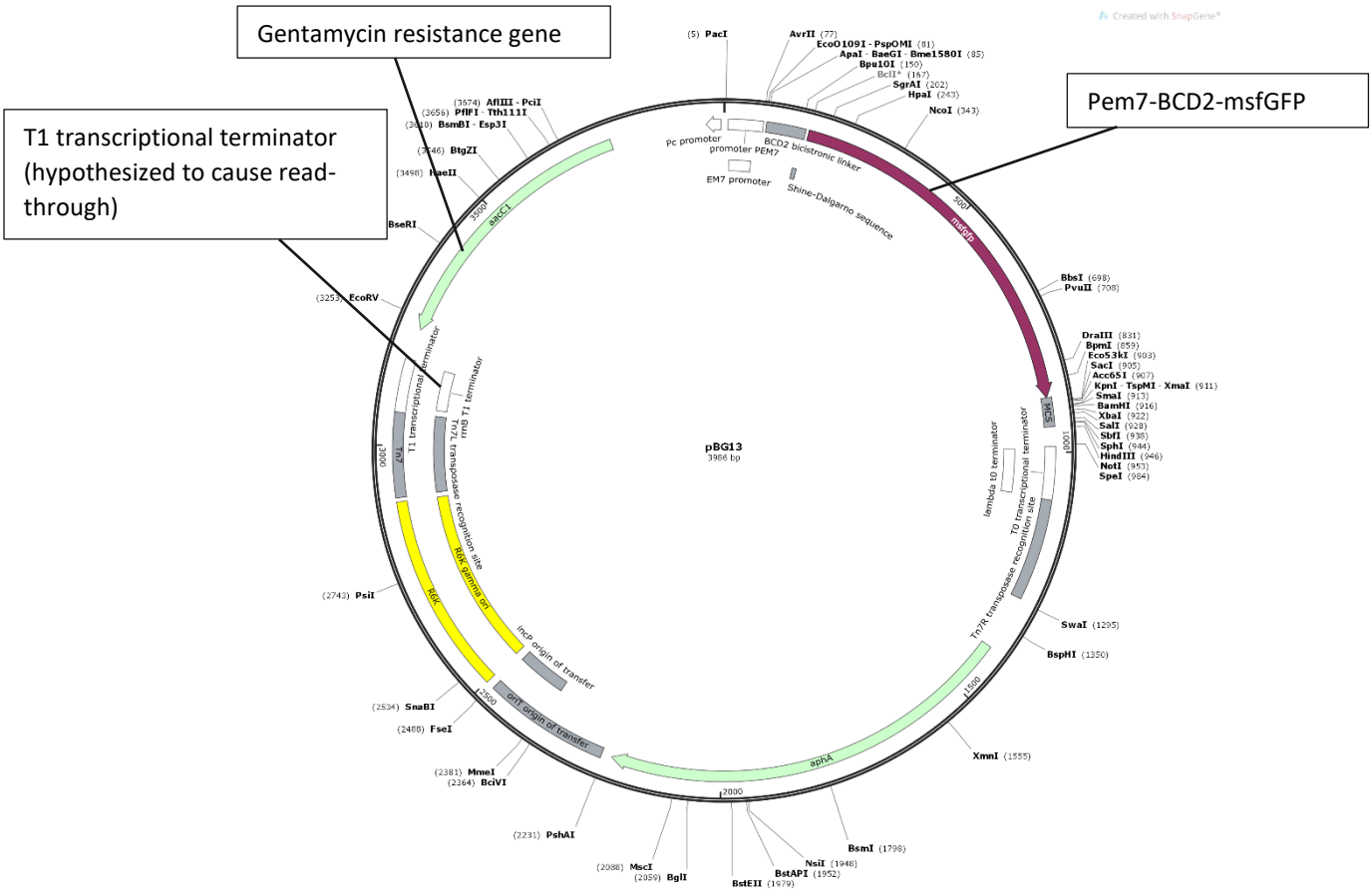

Figure 16. The pBG13 vector map

The pBG13 vector (3986 bp) with the most relevant components indicated. In the modified pBG13 vector, the BCD2-msfGFP section is inverted. Aberrant transcription of the msfgfp gene is thought to proceed from the promoter in front of the gentamycin antibiotic resistance gene.

Discussion

In this research, the goal was to develop a system that could be used to reliably test novel bacteriophage integrases in *P. aeruginosa* with the aim to expand the toolkit of DNA-modifying enzymes for this bacterium. To this end, several options have been explored. First, two variations of a 3-plasmid system were set up in which a constitutive promoter is integrated in front of the *msfgfp* gene. Second, a '5-in-1-plasmid' system was devised in which an inverted *msfgfp* gene is re-inverted by the integrase. However, as technical difficulties hindered the advancement of both of these systems, development of another system (also based on inversion) is currently in progress.

Requirements for a good test system

There are several requirements for a valid test system (**Table 7**). For example, the output must be unambiguous and easily measured. Therefore, in all systems the outcome of recombination involves the onset of msfGFP-fluorescence, which is a clear and reliable output. On top of that, recombination can be confirmed at the DNA level with PCR using well-selected primer pairs. Another necessary aspect of a valid test system is the ease of construction, which is important for high-throughput screening of a range of novel integrases. In this regard, the 5-in-1-plasmid system would require too much tailoring for each integrase and has therefore not been further pursued. Although the cloning of the 3-plasmid system is comparatively uncomplicated, the system may face more practical problems. For example, it may be more difficult for the integrase to locate and recombine the two att-sites when they are more spatially separated. The final system combines the convenient att-site positioning and inversion-related output of the 5-in-1-plasmid system with a less demanding installation resembling the 3-plasmid system. An unexpected finding during the development of this system was the msfGFP-expression in *E. coli* prior to integrase-mediated reversion of the inverted sequence. It has been postulated that read-through by the gentamycin resistance gene caused this. Importantly, this will not be an issue within *P. aeruginosa*, as the promoter that possibly causes the read-through will be directed away from the gene after integration.

Table 7. The advantages and challenges of the various test systems

Each of the three test systems described in this work has their own stronger and weaker points. By carefully weighing the aspects of each system, a thoughtful selection of the best system is possible.

	3-plasmid systems	**5-in-1-plasmid system**	**Modified pBG13 vector**
Reliable output	√ msfGFP expression	√ msfGFP expression	~ msfGFP expression already active in *E. coli* without the integrase
Ease of construction	√ Cloning is feasible ~ Toxicity due to the combination of (leaky) integrase expression and the multiple-antibiotic selection	X SEVAtile cloning of too many (short) tiles	√ the modified pBG13 vector only needs to be made once (starting point further upstream) √ traditional restriction-enzyme cloning
Practical use	X Physical separation of the att-sites may lower recombination efficiency	√ Less influence from plasmid-associated factors (plasmid maintenance, copy-number differences, antibiotic selection)	√ Less influence from plasmid-associated factors (plasmid maintenance, copy-number differences, antibiotic selection)

Another aspect of a good test system is that the integrases used to validate the system are representative for the future candidate integrases. In this research, the integrases phiCTX (Tyr-integrase) and Bxb1 (Ser-integrase) are employed as test-integrases, while the identified candidates are all Tyr-integrases (Gutiérrez et al., 2024). It is therefore more important to optimize the test for phiCTX than for Bxb1. Nonetheless, an optimal system should also be suited for the testing of Ser-integrases so that, once identified for *Pseudomonas*, these integrases can also be tested. A distinctive feature of Tyr-integrases is their dependency on additional factors for efficient recombination (Miyazaki & van der Meer, 2013). In the case of the *Pseudomonas*-derived phiCTX integrase and the current candidate integrases this will not pose a problem, as all factors needed for integration are encoded by the host *P. aeruginosa* strain. Regardless, it means that the Tyr-integrase-associated attP-sites should be estimated broadly to include both the specific sequence recognized during recombination as well as the surrounding regions, which may contain e.g. IHF-binding motifs. In a previous study, mutagenesis

of the att-sites of serine integrase Bxb1 yielded variants with significantly improved integration-rates could be engineered for higher activity and to retrieve the shortest necessary sequences to enhance the efficiency and programmability of integration (Yarnall et al., 2022).

Reported challenges of the different test systems

The integrase toxicity assays performed for the two variations of the 3-plasmid system (with the integrase on pDes1 or pDes4), demonstrated that the integrase could be toxic if it would be too abundantly expressed. The toxicity of pDes1-int appeared remarkably higher than that of pDes4-int, which can be explained by its low copy-number and by the more stringent expression of the rhaB-promoter compared to the XylS/Pm inducible system. Despite this, the poor bacterial growth in the liquid cultures was still observed with the integrase on pDes4. This is thought to be due to a combination of factors, including the multiple antibiotic selection and the growth in minimal media. As all systems include one plasmid with the integrase gene, it is advisable to henceforth always use the pDes4 vector for this and to be mindful of the challenges and risks associated with the liquid culturing of bacteria in all subsequent systems. The use of less systems may be advantageous.

A theoretical disadvantage of the 3-plasmid system, in which the attP-site is already integrated and the attB-site is provided is that, if this attB-site is present somewhere in the genome, the recombinase may inadvertently recombine the inserted attP-site with the natural attB-site instead of the plasmid-borne attB-site. This would lead to an irreversible and catastrophic chromosomal rearrangement, which is detrimental for the bacterium. In theory, this could be averted by first assessing the location of the natural attB-site as opposed to the attP insertion-site (here: the GlmS gene). However, as the genome is not packaged linearly, two genomically distant att-sites may still spatially end up in close proximity to one another. This problem is solved in the system in which the inserted plasmid contains both att-sites, as they would then be in more close proximity to each other.

The 5-in-1-plasmid system and modified pBG13 vector were designed to overcome the possible issues of the 3-plasmid system described above. However, this system evidently faced significant technical challenges in its development. This was not expected, as in the demonstration of SEVAtile cloning combined restriction-ligation was achieved for up to seven tiles (Lammens et al., 2022).

Back to the drawing board: other possible test systems

As things stand, despite the ongoing advancement of the final modified pBG13, no functioning test system has been developed and validated. It is therefore prudent to keep the options open and explore the potential of other test systems. Indeed, the variation of integrase test systems extends far beyond the two main systems discussed in this report.

Another option for a test system with inversion as an output, is to invert the constitutive promoter instead of the gene. However, the att-sites flanking the short promoter sequence may be in too close proximity to each other to allow proper formation of the synaptic complex. This may be solved by inverting a larger segment along with the promoter. Regardless, the SEVAtile cloning would likely still be an issue. It may, however, still be worthwhile to investigate other options to restrict and ligate multiple tiles in one reaction. Perhaps the use of other restriction enzymes, varying the amounts of each component, or adjusting the PCR settings would work. Besides this troubleshooting of SEVAtile, it might also be possible to try a different DNA engineering method, for example USER™ Cloning. In this method, the overlaps provided for the different tiles resemble position markers ending with a deoxyuridine (dU) residue, which can be excised by the USER-enzyme (Uracil-Specific Excision Reagent). This method is reportedly also able to “directionally assemble multiple PCR products into a vector” (Bitinaite et al., 2007). One interesting feature of this system compared to the SEVAtile system is that the overhangs are longer than the SEVAtile position markers (typically 6-10 bp), which infers that this reaction can proceed without a ligase.

Further diversification of the integrase toolbox in P. aeruginosa

Once a successful test system is established, different novel integrases can be tested with it. These candidate integrases and their att-sites are often, including the candidates of this lab, retrieved from bioinformatic analyses of bacterial and/or phage genomes. It may be possible to expand the potential of integrases even further by adjusting the ability of established integrases to recognize a larger library of att-sites (Bland et al., 2017). This would allow for the (re)construction of multiple-gene-cassettes in which recombination will insert all genes belonging to a specific metabolic pathway together at one

genomic locus, using an array of distinct modified attP- and attB-sites (Colloms et al., 2014). Another way to increase the versatility of existing bacteriophage integrases would be to create 'chimeras' of two integrases, which would recognize chimeric att-sites (Farruggio & Calos, 2014b).

Another expansion of the system may be to reverse the recombination. This could be applied to periodically 'reset' memory circuits, e.g., those used in bacteria that sample the inside of bioreactors. Excision requires both the integrase and an additional factor: the excisionase Xis (Tyr-integrases) or recombination directionality factor RDF (Ser-integrases). These factors may be delivered on a separate plasmid (*in trans*, Bland et al., 2017). Since they are phage-encoded, a large-scale genome mining effort will be needed, similar to the one already performed for the current integrases (Guitierrez et al. 2023). Furthermore, the corresponding integrases and their attL- and attR-sites must be known to accurately perform recombination.

Future direction of integrase use and downstream applications

It is important to consider the different requirements for an integrase test system and a context in which the integrase is effectively applied. For a test system of this kind, the simplicity of the overall design is crucial to allow for reliable high-throughput testing of various integrases. Once the validated integrases move to the application phase, more sophisticated additions can be considered. For example, the output will change from msfgfp to a more industrially relevant gene-of-interest (GOI). Many plasmids that are integrated into the genome contain FLP recombination sites (FRT-sites) around the antibiotic resistance gene to remove this gene after successful integration, as antibiotic selection to maintain the plasmid would no longer be necessary (Schweizer, 2003). The removal of antibiotic resistance genes is especially important when the system is ready to move into industry, where the use of multi-drug resistant bacteria is discouraged. Moreover, this is another reason to prefer inversion over insertion as an outcome of recombination, because it would involve less plasmids with antibiotic resistance genes.

Another feature that may change when the integrases will be put to use in the industry is the way in which the 'insertion site' is prepared. In test systems, the (pBG13-)vector can be integrated by the well-established site-specific Tn7-transposon (Choi & Kim, 2009). If later use of the integrase would involve the insertion of multiple genes in a row, it would be needed to pre-insert a concatenation of att-sites into the genome. This may be done in one go using the same transposon. Next, a fusion of a Cas9-gRNA with a reverse transcriptase with the desired att-site to be integrated could be used for the removal or addition of individual att-sites. This type of att-site integration, followed by integrase-mediated recombination has already been successful in human cell lines using Ser-integrases, including Bxb1 (Yarnall et al., 2022). This type of guided integration of att-sites is also applicable when the desire is to integrate a gene at another location within the genome.

Final remarks and future perspectives

In conclusion, this research has underlined the need for more tools for the genetic engineering of Pseudomonas species. Although the original aim of this research, the establishment of a functional integrase test system, has not been met, the engineering and designing process did provide deeper insight into the feasibility of such a system. Given the advantages of a modified vector with an already inverted msfGFP gene, this system will first be further developed for CTX and Bxb1 integrases and validated in PAO1 with the rhaB expression system. If this test system is also ineffective, other test system variations will need to be thoroughly studied and evaluated before a well-informed decision can be made. Eventually, once the test system has been established, the high-throughput screening of novel Pseudomonas-specific bacteriophage integrases can start. This will significantly help to advance the genetic engineering of this non-model organism.

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All explanatory figures in this work were made using Biorender (https://www.biorender.com/). The title page figure represents a lambda phage tyrosine integrase recombining DNA (Holiday junction is shown). It is made by Laguna Design, Science Photo Library (https://www.mediastorehouse.com/science-photo-library/bacteriophage-dna-recombination-f006-9554-9255949.html).

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Layman's summary

By Meira Zubčević

Cut-and-paste

How viruses can help us work with bacteria to build a sustainable future

In recent years, reducing the environmental impact of various industries has become increasingly important. The **genetic engineering*** of bacteria can help to meet this need. Engineered bacteria have many sustainable applications, including drug manufacturing, producing biofuels, and bioremediation (e.g., cleanup of oil spills). To date, researchers have most intensively studied one bacterial species, *Escherichia coli (E. coli)*, because this bacterium is easily cultured and readily accessible. However, *E. coli* does not always function optimally in the harsh environments of bioreactors and other future industrial applications. Other bacteria may be much more suitable for this. One example is *Pseudomonas aeruginosa*, a versatile bacterium with a rich and adaptive metabolism that is much more stress-resistant than *E. coli*. Yet, despite its potential, the lack of genetic engineering tools make it challenging to work with this bacterium. This report discusses a new type of tool to engineer *P. aeruginosa*: **integrases**.

**For the explanation of the words written in bold, see the next page.*

Bacterial invaders

This new tool is borrowed from bacteria's biggest enemies: **bacteriophages**. These bacterial eaters are viruses that specifically infect bacteria to replicate themselves. This often leads to the rupture and death of the bacterium. Sometimes, however, it may be advantageous for a bacteriophage to 'hide out' inside their host, for example when there are not many other bacteria around to infect. To do this, the bacteriophage uses an **integrase** to *integrate* its own DNA into that of its host. This integration happens at a specific location in the genome and in a very precise, controlled manner, without the loss of DNA.

'Cut-and-paste' into the bacterial genome

If bacteriophages can use integrases to 'cut-and-paste' their DNA into that of the bacterium, these integrases could also be used as engineering tools. Scientists would of course not use integrases to cut-and-paste virus-DNA, but to introduce new genes into the bacteria. This **genetic engineering** will let the bacteria produce industrially relevant products, such as vaccine components or enzymes that play a role in plastic degradation.

Exploring 'viral dark matter'

Since there is currently only one *P. aeruginosa*-associated integrase available, scientists are looking for more integrases in nature. A recent study found several integrases by analyzing the largely undescribed regions of bacteriophage DNA, known as 'viral dark matter'. The next step has been to establish a suitable system for the high-throughput testing of these candidate integrases. Two already described integrases, one from *P. aeruginosa* and one from a different bacterium, were used as controls. If the system works for these two controls, it is good enough to be used for the testing of the new integrases.

Testing the test systems

The integrase test systems discussed in this work were based on a **marker gene** in the genome. This gene encodes a fluorescent protein. When the integration is successful, the fluorescence from this protein can be measured. The first systems were designed to integrate a 'gene activator' (**promoter**) in front of the gene. These were fully developed and tested, but no integration occurred. Next, two other systems were designed. In these systems, the marker gene would already be present in the genome, but it would be inverted, like a word in which the letters are upside down and in the reverse order. The integrase causes the gene to 'flip' and makes it readable again.

When this system will finally be established, it will be a useful method to test the efficiency of new integrases for *P. aeruginosa* and other promising bacterial 'work-horses'. This would expand the toolbox for the genetic engineering of these bacteria and prime them as our partners in human health and the sustainable care of the planet.

Difficult words

Genetic engineering	A field of research that applies current knowledge about DNA to the modification of the DNA in certain (micro-)organisms to give them properties that they did not have before
Integrases	Special proteins that can insert, excise, and invert pieces of DNA
Bacteriophage	A type of virus that specifically infects bacteria
Marker gene	A specific gene that is used in research as a sign to see if integration has been successful
Promoter	This is a part of DNA that does not code for a protein, but plays a role in the activation of genes

Supplementary material

Abbreviation convention
Plasmid constructs (before transformation into *E. coli* or *P. aeruginosa*):
vector-insert(s)_phage, e.g. pDes1-int_CTX
For clarity, the 'attP-BCD2-GFP' and 'Pem7-attB' inserts become 'attP' and 'attB', respectively.
The 5-tile plasmid 'pBG13-Pem7-attB-[reverse BCD2-GFP]-(reverse) attP' becomes 'pBG13-attP_*phage*' for the forward direction, and 'pBG13-Ptta_*phage*' for the reverse direction of attP.

Table S1. List of SEVAtile position markers
These are the markers used in the SEVAtile system.

Position marker	Sequence	Specifics
PM1	N-TCTA-N	
PM2	N-GCAG-N	
PM3a	N-CATG-N	Startcodon
PM3b	N-AATG-N	Startcodon
PM4	N-TAAC-N	Stopcodon
PM5	N-GGCT-N	
PM6	N-TAAT-G	Startcodon
PM7	N-TAAGA-N	Stopcodon

Tables S2-S6. List of primers used in this work
A comprehensive list of primers used in this study, categorized based on usage.

Table S2. SEVAtile primers
The primers specifically used in the SEVAtile system. These were used for colony PCR checks or for the formation of the new pSTEntry vector with PM3a-Pem7-PM2.

SEVA_PS1_F	AGGGCGGCGGATTTGTCC
SEVA_PS2 _R	GCGGCAACCGAGCGTTC
InsertDes1_F (pDes1 insert)	AATCTGGCTCCCCAACTAATGC
InsertDes2_F (pBG13 insert)	GCGTTCGGTCAAGGTTCTGGAC
InsertDes4_F (pDes4 insert)	CGGCGAAATAGTAATCACGAGGTCAG
SMZ_ST_Pem7_PM3a_F	TCGGCTCTTCCAGAGGTCTCTCATGTTGTT GACAATTAATCATCGGC
SMZ_ST_Pem7_PM2_R	ATAGCTCTTCACTTGGTCTCGCTGCGGTTT AGTTCCTCACCTTGTC

Table S3. Short primers for the three-plasmid system
*The short primers meant for PCR amplification as used in the three-plasmid system. Restriction sites (RS): GGTCTC (BsaI), GAAGAC (BpiI). Position markers are indicated and start one or two nucleotides after the RS for BsaI and BpiI respectively. *These primers were used both before and after the plasmid-switch. §This is the same primer as in Table S4 and can be used for both systems.*

Position marker	Name	Sequence
pDes4		
PM4	MB_pSTD4_BsaI_F	GACGGTCTCCTAACAACTAGTCTTGGACTCCTGTTG
PM1'	MB_pSTD4_BsaI_R	GTCGGTCTCCTAGAGAATTTCATTACGACCAGTC
PM4	MB_pSTD4_BpiI_F	GACGAAGACACTAACAACTAGTCTTGGACTCCTGTTG
PM1'	MB_pSTD4_BpiI_R	GTCGAAGACACTAGAGAATTTCATTACGACCAGTC
PM1	MB_pem7_BpiI_F	GACGAAGACATTCTATTGTTGACAATTAATCATCGGCATAG
PM2'	MB_pem7_BpiI_R	GTCGAAGACCACTGCGGTTTAGTTCCTCACCTTGTC
PM2	MB_CTXattB_2_BsaI_F	GATGGTCTCAGCAGGTACTATGGCGCCCCGCGATG

PM4'	MB_CTXattB_4_BsaI_R2	GAAGGTCTCAGTTACAGGCTGCGGAAACGACC
PM1	MB_phiCTX_PM1_pD4_F	GATGGTCTCATCTATGGCAGACGGAGTCGAG
PM1	SMZ_intBxb1_pDes4_F	GATGGTCTCATCTATGAGAGCCCTGGTAGTC
	pDes1	
PM3a	MB_phiCTX_pDes1_F	GATGGTCTCACATGGCAGACGGAGTCGAG
PM4'	MB_phiCTX_pDes1_R*	CTGGGTCTCAGTTATCAGGAAAGTTTCAGCGCCTG
-	MB_intCTX_pDes1_R2*	GACGCCGTTGGATACACCAAG
PM3a	MB_Bxb1_int_pDes1_F	GATGGTCTCACATGAGAGCCCTGGTAGTC
PM4'	MB_Bxb1_int_pDes1_R*	CGAGGTCTCAGTTACTACGACATCCCGGTGTG
	pBG13	
PM7	MB_pBG_BPiI_F	GATGAAGACCTAAAGAGAATTCGAGCTCGGTAC
PM1'	MB_pBG_BPiI_R	CACGAAGACCATAGATTAATTAAGACGTCTTGACATAAG
PM1	MB_BpiI_attP_PM1 _F	GACGAAGACATTCTATCAGCAAGGACGGCCCCGAC
PM2'	MB_ BpiI_attP_PM2_R	GTCGAAGACCACTGCTTTCAATGGGTCAACATGAAATC
PM3b	SMZ_GFP_BsaI_F§	GTCGGTCTCAAATGATCATGGGAATTCATAAAGG
PM7'	SMZ_GFP_BsaI_R	GTCGGTCTCAAAGATTATTTGTAGAGTTCATCCATGCC

Table S4. Short primers for the 5-in-1-plasmid system

The short primers meant for PCR amplification as used in the 5-in-1-plasmid system. Restriction site (RS): GGTCTC (BsaI). Position markers are indicated and start one nucleotide after the RS. §This is the same primer as in Table S3 and can be used for both systems.

Position marker	Name	Sequence
	pBG13	
PM4	SMZ_GFPrv_PM4_F	GTCGGTCTCATAACTTATTTGTAGAGTTCATCCATGCCG
PM3b	SMZ_GFPrv_PM3b-r_R§	GTCGGTCTCAAATGATCATGGGAATTCATAAAGG
PM3b'	SMZ_BCD2rv_PM3b-r_F	GTCGGTCTCACATTAGAAAACCTCCTTAGCATGA
PM5	SMZ_BCD2rv_PM5-r_R	GTCGGTCTCAGGCTGCCCAAGTTCACTTAAAAAGG
PM5'	SMZ_CTXPtta_PM5-r_F	CATGGTCTCAAGCCTTTCAATGGGTCAACATGAAATCC
PM5'	SMZ_CTXattP_PM5-r_F	CATGGTCTCAAGCCTCAGCAAGGACGGCCCCGACATC
PM7'	SMZ_CTXattP_PM7_R	CATGGTCTCATCTTTTTCAATGGGTCAACATGAAATCC
PM7'	SMZ_CTXPtta_PM7_R	CATGGTCTCATCTTTCAGCAAGGACGGCCCCGACATC

Table S5. Long primers for primer annealing

*The long primers used for primer annealing, for both systems. Restriction sites (RS): GGTCTC (BsaI), GAAGAC (BpiI). Position markers are indicated and start one or two nucleotides after the RS for BsaI and BpiI respectively. Some primers do not have any an RS, because these already have overhangs after annealing. *These primers were used for the formation of a 10 bp insert in the pDes1-vector for the negative control in the pDes1-int system.*

Position marker	Name	Sequence
	three-plasmid system	
PM3a	MB_CATG-rdm-ins_1*	CATGGACGGTACTG
PM4'	SMZ_GTTA_rdm-ins_2*	GTTACAGTACCGTC
PM2	MB_Bxb1_attB_pDes4_F	GATGAAGACTAGCAGGCTTGTCGACGACGGCGGTCTCCGTCGTCA GGATCATTAACTGGTCTTCTTC
PM4'	MB_Bxb1_attB_pDes4_R	GAAGAAGACCAGTTAATGATCCTGACGACGGAGACCGCCGTCGTC GACAAGCCTGCTAGTCTTCATC
PM1	MB_Bxb1_atP_pBG_F2	GACGAAGACATTCTAGTGGTTTGTCTGGTCAACCACCGCGGTCTCA GTGGTGTACGGTACAAACCCAGCAGTGGTCTTCTAC
PM2'	MB_Bxb1_atP_pBG_R2	GTAGAAGACCACTGCTGGGTTTGTACCGTACACCACTGAGACCGC GGTGGTTGACCAGACAAACCACTAGAATGTCTTCGTC

PM2	SMZ_BCD2_PM2_F	GCAGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATT TTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT
PM3b'	SMZ_BCD2_PM3b_R	CATTAGAAAACCTCCTTAGCATGATTAAGATGTTTCAGTACGAAAAT TGCTTTCATTGTTGATCTCCTTTTTAAGTGAACTTGGGC
5-in-1-plasmid system		
PM5'	SMZ_attP_r_Bxb1_1	GACGAAGACATAGCCTGGGTTTGTACCGTACACCACTGAGACCGC GGTGGTTGACCAGACAAACCACAAGATGGTCTTCTAC
PM7'	SMZ_attP_r_Bxb1_2	GTAGAAGACCATCTTGTGGTTTGTCTGGTCAACCACCGCGGTCTCA GTGGTGTACGGTACAAACCCAGGCTATGTCTTCGTC
PM5'	SMZ_attP_f_Bxb1_1	GACGAAGACATAGCCGTGGTTTGTCTGGTCAACCACCGCGGTCTC AGTGGTGTACGGTACAAACCCAAAGATGGTCTTCTAC
PM7'	SMZ_attP_f_Bxb1_2	GTAGAAGACCATCTTTGGGTTTGTACCGTACACCACTGAGACCGCG GTGGTTGACCAGACAAACCACGGCTATGTCTTCGTC

Table S6. Primers for the modified pBG13 vector
*All primers used for the modified pBG13 vector. Restriction sites: GGATCC (BamHI), CCTAGG (AvrII), AAGCTT (HindIII) *These primers have only been designed and ordered, but they have not yet been used in this study.*

Name	**Sequence**
SMZ_BamHI_BCD2_F	GTAGGATCCGCCCAAGTTCACTTAAAAAGGAG
SMZ_AvrII_GFPrv_R	GTACCTAGGCCGGGTACCGAGCTCGAATTC
SMZ_AvrII_attBCTX_F	GTACCTAGGGTACTATGGCGCCCCGCGATG
SMZ_AvrII_attBCTX_R	GTACCTAGGCAGGCTGCGGAAACGATCCGGAAG
SMZ_AvrII_attBBxb_F	CTACCTAGGGCTTGTCGACGACGGCGGTCTCC
SMZ_AvrII_attBBxb_R	GTACCTAGGATGATCCTGACGACGGAGACCGCC
SMZ_BamHI_attPCTX_F	GTCGGATCCTCAGCAAGGACGGCCCCGACATC
MB_HindIII_atPCTX_R	GTCAAGCTTTTTCAATGGGTCAACATGAAATCC
SMZ_BamHI_PttaCTX_F	CATGGATCCTTTCAATGGGTCAACATGAAATCC
MB_HindIIIPttaCTX_R	GTCAAGCTTTCAGCAAGGACGGCCCCGACATC
SMZ_BamHI_attPBxb_F	GTAGGATCCGTGGTTTGTCTGGTCAACCACCG
MB_HindIII_atPBxb_R	GTCAAGCTTTGGGTTTGTACCGTACACCACTGAG
SMZ_BamHI_PttaBxb_F	GTCGGATCCTGGGTTTGTACCGTACACCACTGAG
MB_HindIIIPttaBxb_R	GTAAAGCTTGTGGTTTGTCTGGTCAACCACCG
MB_attB-ins-check_F*	AGGGCGGCGGATTTGTCC
MB_attB-ins-check_R*	GTTGAAGACGGTAGCGTACAGCTGG
MB_attP-ins-check_F*	CCAGCTGTACGCTACCGTCTTCAAC
MB_attP-ins-check_R*	GATAAATGGTCGCGCCGCTCTGACC

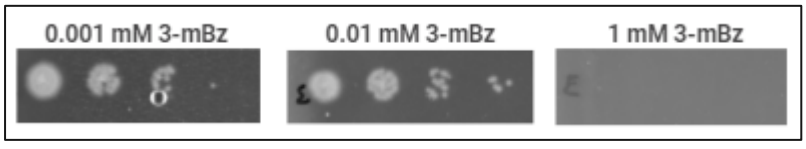

Figure S1. Additional 3-mBz concentrations tested for phiCTX toxicity
The colonies of 'spots', serial tenfold dilutions from bacterial cultures that had reached an OD_{600} of ~0.3. The bacteria that were induced are PAO1:pBG13-attP:pDes4-attB+pDes1-int_CTX. When the bacteria were induced with 1 mM 3-mBz, there was complete toxicity. 3-mBz: 3-methylbenzoate

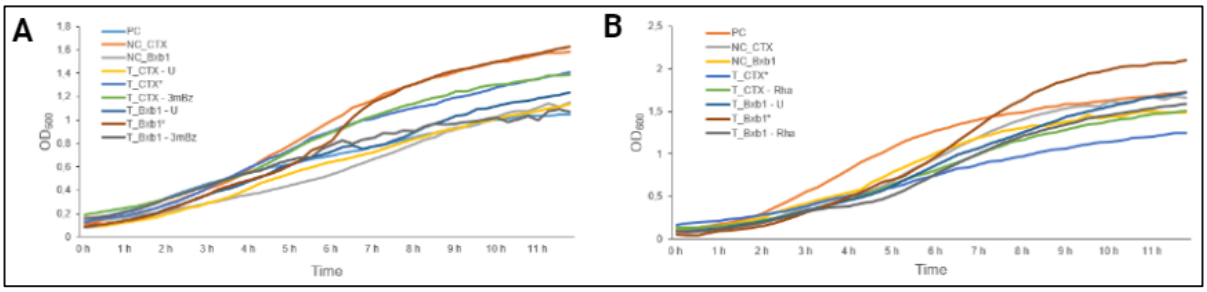

Figure S2. Growth curves during the induction experiment

*These are the OD_{600} values as measured over time on the CLARIOstar (BMG Labtech) during the induction experiment. A) Induction with 3-mBz for the XylS/Pm expression system (with the integrase on pDes1), B) Induction with Rha for the rhaB-promoter expression system (with the integrase on pDes4). For more information on experimental settings, see the caption of Figure S3. PC: positive control, NC_CTX/Bxb1: negative control, these are different for the two different induction systems, T_CTX/Bxb1: the test strains that were transformed with all three plasmids (attP, int, and attB), *These test strains had previously been induced on plates. 3mBz: 3-methylbenzoate, Rha: rhamnose*

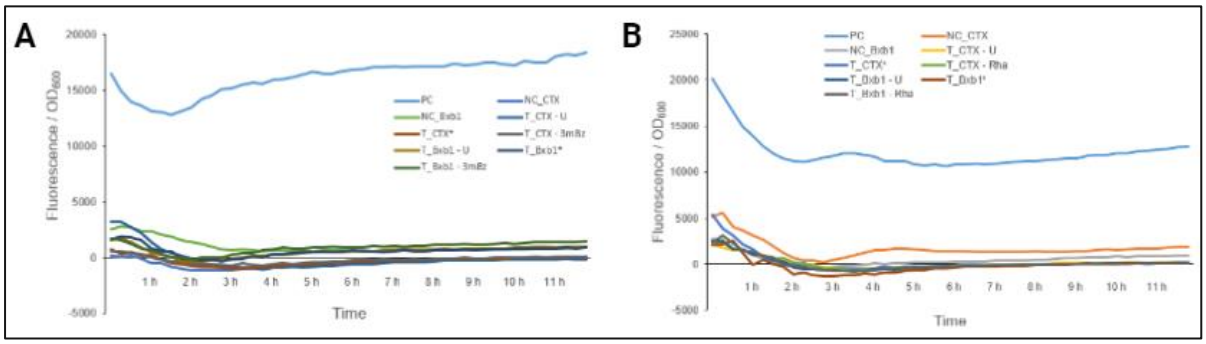

Figure S3. Fluorescence intensity after induction of PAO1 strains in the 3-plasmid system

*Graphs showing the fluorescence intensity at the GFP emission wavelength over time, as recorded in the CLARIOstar (BMG Labtech). A) Induction with 3-mBz for the XylS/Pm expression system (with the integrase on pDes1), B) Induction with Rha for the rhaB-promoter expression system (with the integrase on pDes4). Experiments were conducted on different days, but all experimental conditions were identical (see Methods). PC: positive control, NC_CTX/Bxb1: negative control, these are different for the two different induction systems, T_CTX/Bxb1: the test strains that were transformed with all three plasmids (attP, int, and attB), *These test strains had previously been induced on plates. 3mBz: 3-methylbenzoate, Rha: rhamnose All data was blank- and OD600-corrected. Data represents the average for triplicates. All samples in the top graph, and NC_Bxb1 and plate-induced samples in the bottom graph represent n=3 technical replicates. The rest of the samples in the bottom graph represent n=3 biological replicates.*

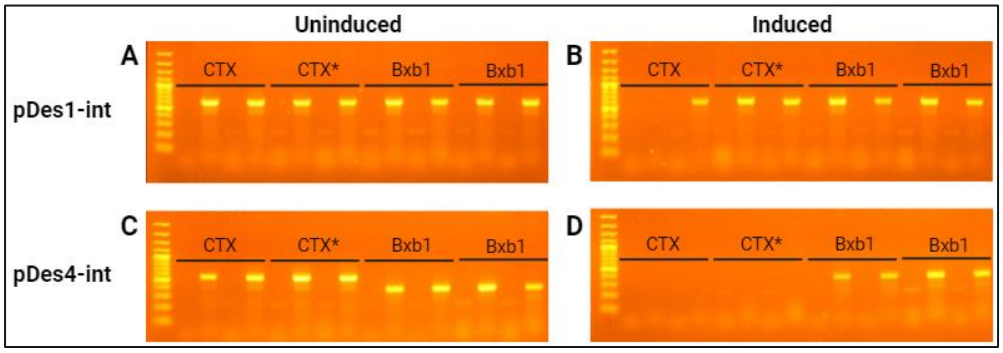

Figure S4. Full gels for the integration check before and after induction: one of the primer-sets did not work

These are the full gels for the recombination assessment in fully transformed PAO1 with the pDes1-int (top) and pDes4-int (bottom) variations of the three-plasmid system. In each gel, the first lane was loaded with the product of the PCR with primer-set P (amplifying attP-GFP before integration and attR after integration), and the second lane was loaded with primer-set B (amplifying Pem7-attB before integration and attL after integration). The PCR with primer-set P did not work here. From the other PCR, it was concluded that recombination did not succeed. Bands remain the expected lengths for before recombination (600 bp for CTX, and 400 bp for Bxb1). All gels are 1.5% gels with the 100bp ladder.

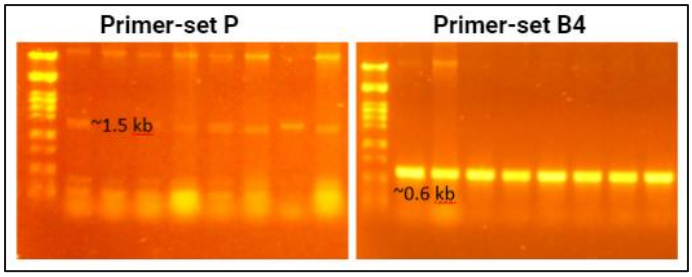

Figure S5. Full gel for the first integration check pDes1-int_CTX
These are the full gels for the recombination assessment after induction of fully transformed PAO1 with the pDes1-int (the three-plasmid system). The bands correspond with the expected lengths before recombination (see main text). Both gels are 1.5% gels with the 100bp ladder.

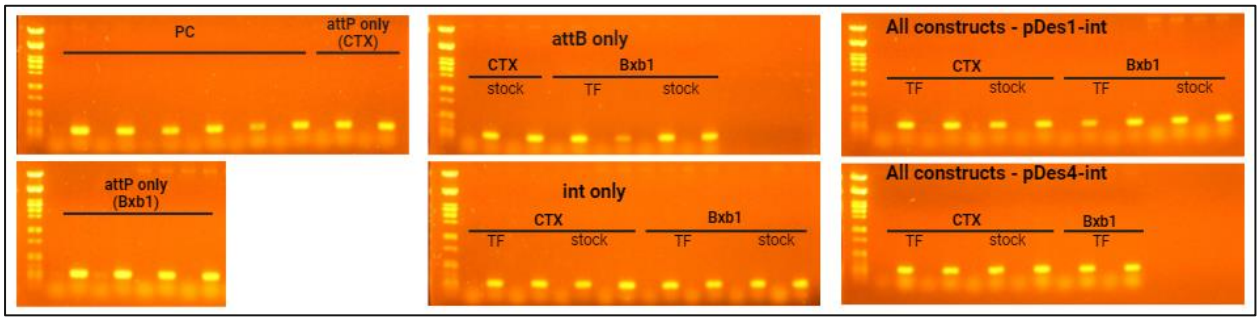

Figure S6. Gels with integration check after the glms-gene
All PCR products shown in Figure XC. Bacterial clones are from all steps in the process, loaded in duplicate. Each first lane represents primer-set P (pDes2_F and PS2_R) and each second lane represents the primer-set GlmsDown and Tn7R, which is supposed to show a low band if something has integrated just after the glms-gene. This shows that indeed something has integrated at the transposase integration site in the PAO1 genome. In this check, the PCR with primer-set P did not work yet. attP only: PAO1:pBG13-attP:CTX/Bxb1, attB only: PAO1:pBG13-attP:pDes4-attB_CTX/Bxb1, int only: PAO1:pBG13-attP:pDes4-int_CTX/Bxb1. All constructs: PAO1 transformed with all three plasmids, with either pDes1-int (top) or pDes4-int (bottom). All gels are 1% agarose gels with the phage lambda DNA PstI digest as a marker.

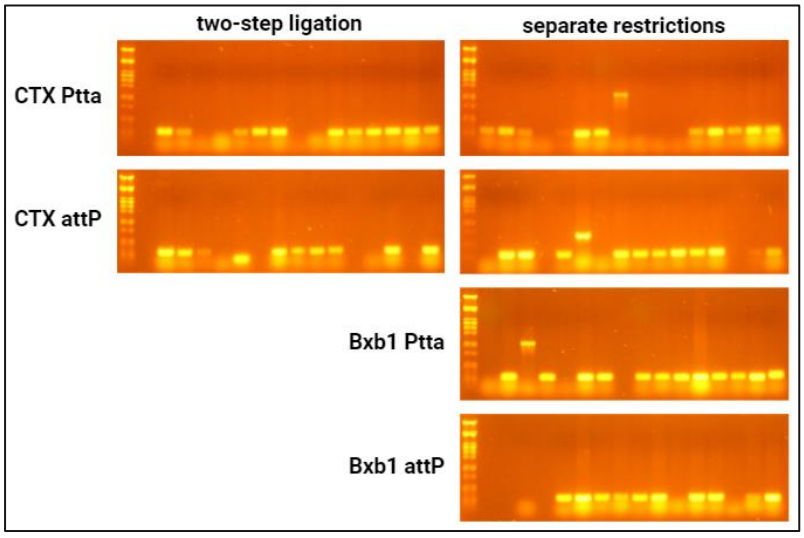

Figure S7. Colony PCR of the 5-in-1 plasmid system
These gels represent the result of the second (and third) transformations for the 5-in-1 plasmid system. This time, for CTX two types of ligation had been attempted, separate restrictions (right) and a two-step ligation in which some fragments were pre-ligated (right, for explanation see main text). From these gel images, it becomes clear that most colonies were transformed with an empty pBG13 vector, indicating that ligation is the most challenging part of this system. Some of these colonies were sequenced (see Figure S8). All gels are 1% agarose gels with the phage lambda DNA PstI digest as a marker.

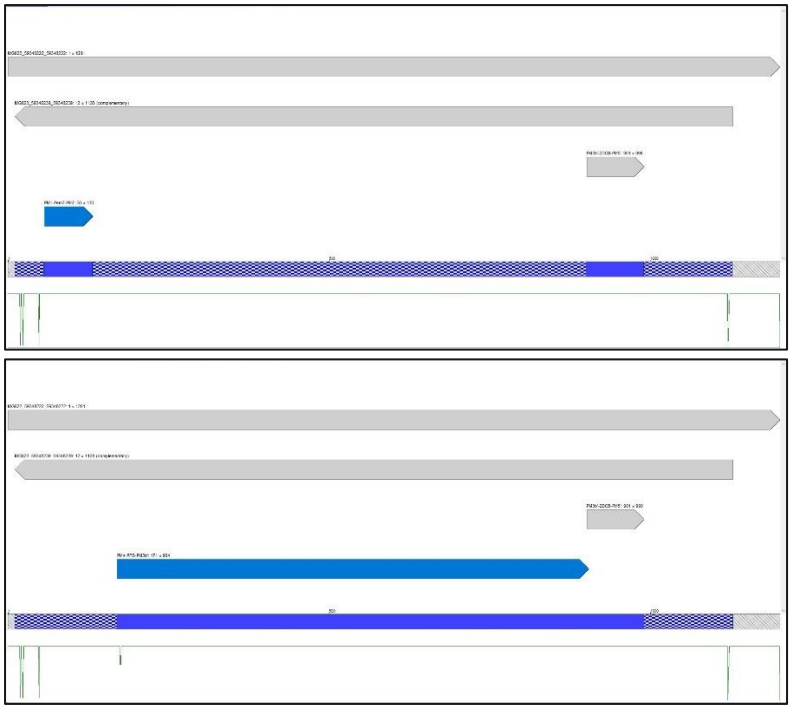

Figure S8. Sequencing results of Bxb1-Ptta in the 5-in-1 plasmid system
Graphical representations of the sequencing results for the Bxb1-Ptta plasmid. In the first attempt (top), only the Pem7 promoter and the reversed BCD2-fragment ('2DCB') were present. In the second attempt (bottom), only the reversed GFP-fragment ('PFG') and the reversed attP-site ('Ptta') were present. For the other five-tile plasmids, no gene-assembly was made. Analysis was done in contigExpress (Vector NTI Advancer 11).

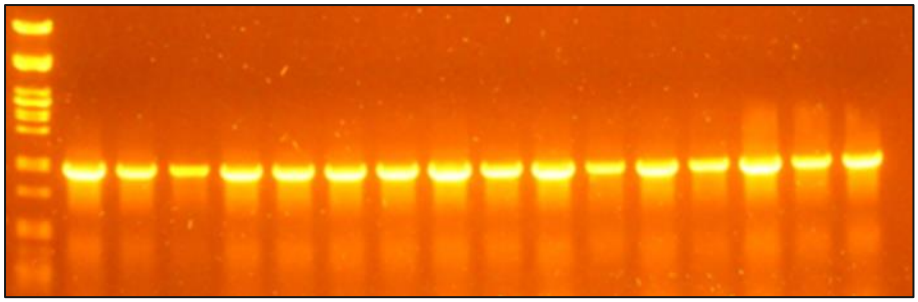

Figure S9. Colony PCR for the modified pBG13 vector
The PCR was done with the primers insert_pDes2_F and SEVA_PS2_R, which would yield the same height both for the original sequence as well as the flipped sequence. Therefore, this PCR only confirmed that the vector was not empty. Inversion was successfully demonstrated with the alignment shown in Figure S10 below. This is a 1% agarose gel with the phage lambda DNA PstI digest as a marker.

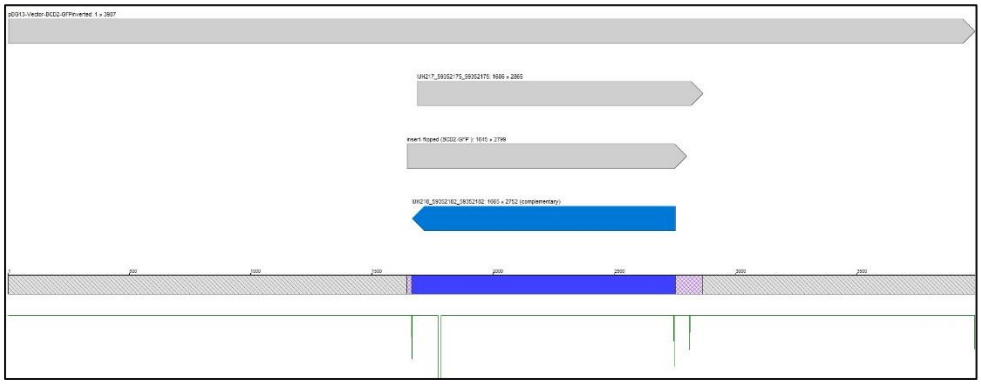

Figure S10. Sequencing result for the modified pBG13 vector

Graphical representations of the sequencing results for the modified pBG13 vector. It is clear that the BCD2-GFP-fragment is inverted. In the first attempt (top), only the Pem7 promoter and the reversed BCD2-fragment ('2DCB') were present. In the second attempt (bottom), only the reversed GFP-fragment ('PFG') and the reversed attP-site ('Ptta') were present. For the other five-tile plasmids, no gene-assembly was made. Analysis was done in contigExpress (Vector NTI Advancer 11).

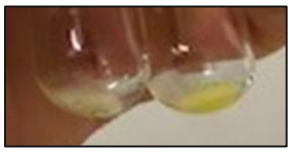

Figure S11. Green fluorescence of the modified pBG13 vector
This photo shows how the bacteria with the original pBG13 vector were not green (left), while those with the modified pBG13 were (right).

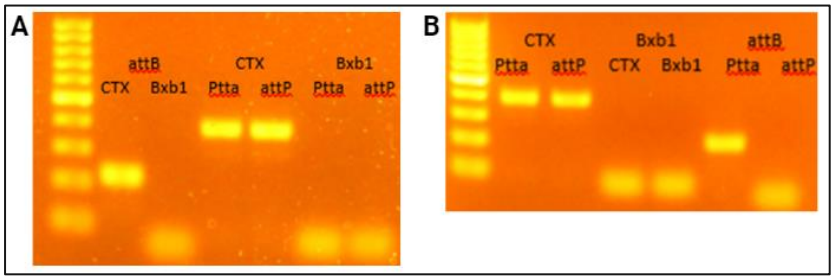

Figure 12 PCR and restriction of attB- and Ptta/attP-sites of CTX and Bxb1
The att-sites were prepared by PCR and restriction. The difference in size is not visible , which is likely because the only difference after restriction is that all inserts have overhangs (AvrII-attB-AvrII and BamHI/HindIII-attP-HindIII/BamHI). Both gels are 1.5% agarose gels with the 100bp ladder.