Chandramowli and Devreese *AMB Express* (2025) 15:34
https://doi.org/10.1186/s13568-025-01844-2

AMB Express

ORIGINAL ARTICLE Open Access

A method for creating in-frame insertions of fluorescent proteins in non-model gram-negative bacteria

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Abstract
The use of fluorescent proteins to study protein expression and localisation has become common practice in the life sciences. While methods to create gene fusions and replacements with fluorescent proteins in model organisms have rapidly developed, there exist far fewer well-established protocols applicable to non-model bacteria. Here, we present a comprehensive account of an allelic-exchange-based mutagenesis strategy using the I-SceI endonuclease in a clinical strain of *S. maltophilia*. We demonstrate the use of this strategy for the creation of in-frame insertions of fluorescent proteins and entire gene replacements for the purposes of studying protein localisation and expression. This protocol requires minimal setup, and once optimised, can produce mutants in a matter of weeks. We expect this strategy to be of use for laboratories working with poorly-characterised strains and/or bacteria for which information is scarce.

Key points
- Genetic manipulation of non-model Gram-negative bacteria can be cumbersome.
- Allelic-exchange mutagenesis has typically been used only for creating deletions.
- We showcase its potential for creating insertions and gene replacements using fluorescent proteins.

Keywords *S. maltophilia*, Allelic-exchange mutagenesis, I-SceI endonuclease, Insertions, Replacements, eGFP, mCherry

Introduction

Even before Francis Crick postulated the central dogma of molecular biology to explain the flow of genetic information in cells, the importance of studying proteins in addition to the instructions that tell the cell how to make them was already recognised (Crick 1970). While the early half of the 20th century is remembered for multiple discoveries leading to the conclusion that DNA is the primary genetic material in all living organisms, developments in the fields of structural and functional protein biology during the same timeframe are relatively overshadowed (Sumner 1926; Sanger 1949; Pauling and Corey 1951; Kendrew et al. 1958; Muirhead and Perutz 1963). However, by the turn of the century, the well-established field of genetics and the still nascent field of proteomics were coming to terms with their complementary natures. Following the success of the Human Genome Project, the cost of genome sequencing steadily decreased, allowing for the generation of significant amounts of genetic data. This facilitated in-depth studies into the physiology and pathogenicity of many clinically-relevant bacteria,

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thereby allowing for the identification and development of better treatment strategies.

When studying a cell's response to stimuli, it is often useful to obtain real-time information not just at the transcriptional and translational levels, but also at the spatiotemporal level. In particular, when examining the response of bacteria to antibiotic stress, studies have shown that in addition to their intended targets, antibiotics can also cause re-localisation of bacterial proteins to different subcellular compartments such as at concentrated foci at the cell membrane or secreted vesicles (Rudner and Losick 2010). This information can be used to determine the efficacy of a certain course of treatment in vitro prior to its administration in vivo, which is especially useful when dealing with multidrug-resistant (MDR) bacteria. The most commonly used reporter genes for expression and localisome analyses are fluorophores, such as the green fluorescent protein (GFP) from *Aequorea victoria* (Prasher et al. 1992). Such genes can be introduced into bacteria to study protein localisation as well as serve as a form of protein quantification, making them an important tool in the arsenal of any microbiologist.

Permanent genetic manipulation of model Gram-negative bacteria, such as *Escherichia coli*, has been achieved through increasingly sophisticated methods including recombineering and CRISPR-based techniques (Sharan et al. 2009; Tischer et al. 2010; Yao et al. 2018). While the scope of these methods is expanding to clinically-relevant bacteria, they are still typically restricted to the type-strains of model organisms. For other bacteria, the traditional approach for genetic manipulation involves allelic-exchange mutagenesis, wherein the desired allele is introduced into the target organism's genome on a suicide vector, following which it is made to replace the endogenous allele via homologous recombination (HR) (Kolisnychenko et al. 2002). Such protocols have already been reported for multiple known pathogens, most notably *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Clostridium*; however, these studies have mainly demonstrated the creation of non-polar and in-frame deletions and to a lesser extent to incorporate point mutations (Flannagan et al. 2008; Zhang et al. 2015; Hmelo et al. 2015).

Here, we present an account of the I-SceI endonuclease-mediated strategy for the genetic manipulation of clinical isolates of Gram-negative bacteria. To this end, we use a non-typical strain of the MDR bacterium *Stenotrophomonas maltophilia* (strain 44/98, LMG 26824) as a model. This strain has previously been used in our laboratory in different contexts (Devos et al. 2015, 2016, 2017); however, prior genetic manipulation attempts with it have been cumbersome and largely ineffective. We report the construction of various fluorescent proteins insertions in-frame of proteins relevant to ongoing studies pertaining to the antibiotic response of *S. maltophilia*. Specifically, we show how our method can be used to tag membrane and cytoplasmic proteins with enhanced green fluorescent protein (eGFP) and mCherry, including the complete replacement of a highly expressed membrane protein with its mCherry-tagged form.

Analysis of I-SceI endonuclease-mediated allelic-exchange

This strategy was initially developed for use in multidrug-resistant strains of *B. cenocepacia* to create scarless deletions (Flannagan et al. 2008; Hamad et al. 2010). Briefly, a suicide plasmid containing a "defective" origin of replication (meaning that it is dependent on a certain protein for replication) and homology regions flanking the target site is introduced into the desired organism, where it integrates into the genome through HR. A second plasmid that constitutively expresses the I-SceI endonuclease is then introduced into a successful transformant (termed an ex-conjugant), resulting in a double-strand break (DSB) at the recognition site which is also introduced on the suicide vector. This break is repaired via the target's HR-mediated machinery, resulting in either reversion to the wild-type (WT) or successful introduction of the mutation (Figs. 1 and 2). Compared to other site-directed mutagenesis methods, this method offers multiple advantages–

- It is a relatively time and resource-efficient alternative when working with organisms for which little information is available– apart from genomic data, only knowledge of basic microbiological and molecular techniques is required.
- There is no need for additional steps, such as excision of antibiotic markers, which would increase the chance of spontaneous mutation.
- Suicide vectors for one strain can often be used for other strains of the same bacterium, making them broadly applicable.

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. *E. coli* was maintained in regular lysogeny broth (LB, LB-Miller), while *S. maltophilia* was maintained in a low-salt LB formulation (LB-Lennox). *S. maltophilia* was also grown on *Pseudomonas* Isolation Agar (PIA) plates for selection of successful transformants. All liquid cultures were grown at 37 °C in aerobic conditions, and agar plates were incubated at either 30 or 37 °C. The following antibiotics were used for selection in or against *E. coli*– trimethoprim at 50 µg/ml, kanamycin at 50 µg/ml, tetracycline at 10 µg/ml, and norfloxacin at 2 µg/ml. The

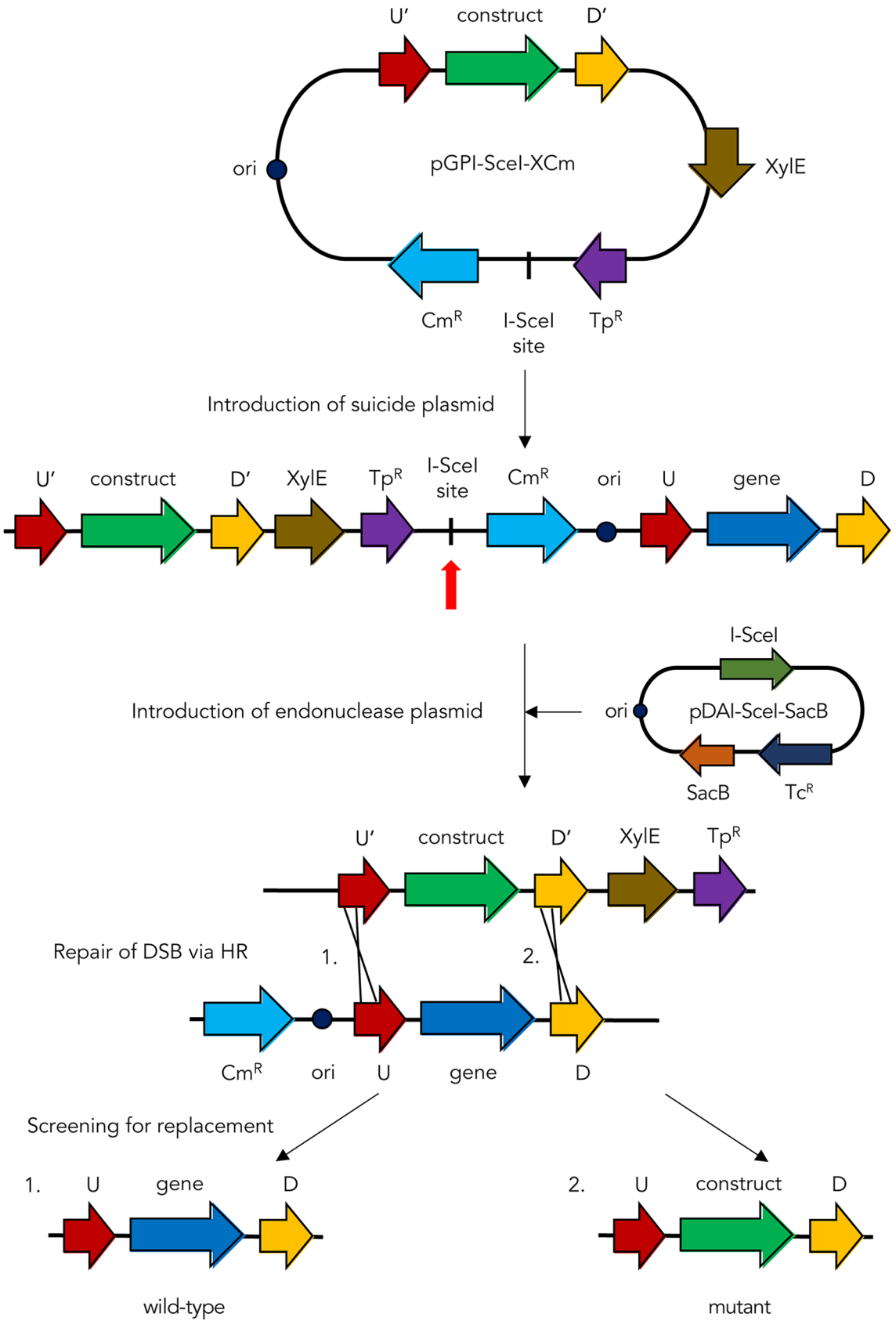

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 A general representation of the I-SceI endonuclease-mediated mutagenesis strategy. The construct of interest flanked by upstream (U') and downstream (D') homology regions is cloned into the pGPI-SceI-XCm suicide plasmid, which also harbours the I-SceI recognition site. This plasmid is introduced into *S. maltophilia* where it integrates into the genome, producing an ex-conjugant. Next, the endonuclease plasmid pDAI-SceI-SacB is introduced into the ex-conjugant, leading to a lethal DSB that needs to be repaired before cell division continues. During repair, there are two possible locations of HR, hence two outcomes– either reversion back to WT (1.) or successful mutation (2). ori– origin of replication; XylE– *xylE* reporter gene; Tp^R– trimethoprim resistance gene; I-SceI site– I-SceI endonuclease recognition site; Cm^R– chloramphenicol resistance gene; I-SceI– I-SceI endonuclease-coding gene; Tc^R– tetracycline resistance gene; SacB– *sacB* counter-selectable gene.

following antibiotics were used for selection in *S. maltophilia*– trimethoprim at 50 µg/ml, chloramphenicol at 40 µg/ml, and tetracycline at 17 µg/ml.

Design of mutant alleles and construction of suicide vectors

The pGPI-SceI-XCm plasmid was used as the backbone to introduce the mutant alleles into the recipient *S. maltophilia*. The inserts were designed depending on the type of mutation required (i.e., insertion or replacement)– the exact process for each is mentioned in the Results section. The sequences were derived from the published genome for the type-strain K279a (Crossman et al. 2008). Previous proteomics experiments in our laboratory have demonstrated that proteins isolated from strain 44/98 were easily identifiable based on genetic information obtained from strain K279a, highlighting the similarity between the genomes (Van Oudenhove et al. 2012). Therefore, we considered that there would be sufficient similarity between the two genomes to allow us to use the reference genome to design our constructs, such that HR would occur. While all plasmids were designed in this study, their syntheses were outsourced to GenScript Biotech (Piscataway, New Jersey).

Transformation of suicide plasmids into *S. maltophilia*

Suicide plasmids bearing the mutant alleles were transformed into *S. maltophilia* through triparental mating (Supplementary Table S1). Briefly, 800 µl of overnight cultures of the recipient *S. maltophilia*, and the donor and helper *E. coli* were collected and washed with fresh LB to remove traces of selective antibiotics. Cell pellets were resuspended and combined in a total of 100 µl of fresh LB, and cells were spotted on a nitrocellulose filter (0.22 µm pore size) placed on an LB agar plate. Once the spot had dried, the plate was incubated at 37 °C overnight to allow for conjugation. The following day, the filter was transferred to a microcentrifuge tube, and cells were dislodged into fresh LB by vortexing. Cells were plated in triplicate on PIA supplemented with trimethoprim and chloramphenicol to select for *S. maltophilia* transformants and norfloxacin to select against *E. coli*. Plates were incubated at 37 °C for 2–3 days to allow colonies to grow. Once colonies had reached sufficient size (around 2 mm diameter), they were screened using a solution of 0.45 M pyrocatechol– colonies that turned yellow were considered true ex-conjugants.

Transformation of pDAI-SceI-SacB into *S. maltophilia*

pDAI-SceI-SacB was transferred to *S. maltophilia* ex-conjugants either through triparental mating (same as above) or electroporation (Supplementary Table S2). Briefly, ex-conjugant cells were grown in LB-Lennox supplemented with 10 mM $MgSO_4$ until they reached mid-exponential phase. Cells were harvested and washed twice with ice-cold 10% glycerol to make them electrocompetent. The cell pellet was resuspended in 1 ml of 10% glycerol and aliquots of 50 µl were made. 100–200 ng of pDAI-SceI-SacB was electroporated into electrocompetent *S. maltophilia* with the following conditions– 25 kV/cm field strength, 25 µF capacitance, and 200 Ω resistance– taking care to ensure a minimum time constant of 5 ms. Cells were allowed to recover in super optimal broth with catabolite repression (SOC) for 3 h at 30 °C with constant shaking. Cells were plated on LB-Lennox supplemented with tetracycline to select for *S. maltophilia* transformants. Plates were incubated at 30 °C for 2–3 days to allow colonies to grow. Once colonies had reached sufficient size (around 2 mm diameter), they were screened by colony polymerase chain reaction (PCR) using appropriate primers (Supplementary Tables S3 and S4). Successful mutants were cured of pDAI-SceI-SacB through repeated subculture in LB supplemented with 5% (w/v) sucrose.

Fluorescence microscopy

Overnight cultures of *S. maltophilia* mutants with fluorescent protein insertions were back-diluted in fresh LB-Lennox at a ratio of 1:50 and allowed to grow for 3–4 h. Meanwhile, agarose pads were prepared using a 1% agarose solution sandwiched between 2 glass coverslips (1.5 thickness). For imaging, the pads were cut into small squares (5 mm x 5 mm) and transferred to another glass coverslip. 10–20 µl of each culture was spotted on a pad and placed on a glass slide (with the bacteria towards the slide). A drop of immersion oil (refractive index 1.52) was placed on the slide before transferring it to the microscope stage.

Samples were visualised using a spinning disk confocal microscope (Nikon Eclipse Ti, Japan) equipped with an MLC 400 B laser box (Agilent Technologies, California, USA), a Yokogawa CSU-X confocal spinning disk device (Andor, Belfast, UK), an iXon Ultra EMCCD camera (Andor Technology, Belfast, UK), and a Plan Apo VC 100 × 1.4 NA oil immersion objective lens (MRD01902,

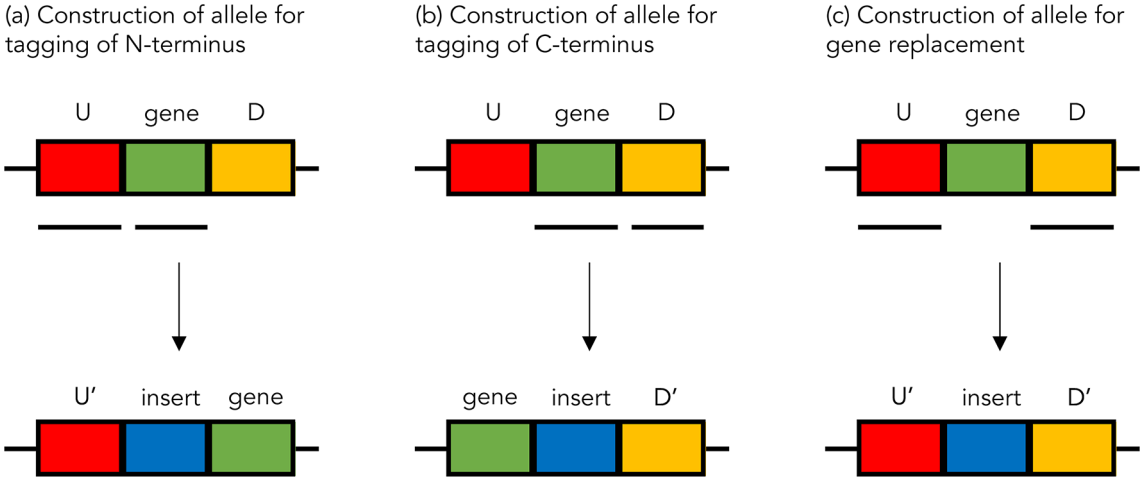

Fig. 2 Construction of alleles for tagging and replacement of endogenous genes. All instances are shown for genes present on the plus-strand. **a** In the case of N-terminal tags, the upstream sequence of the allele (U′) consists of the region immediately upstream of the target gene, while the downstream region consists of a portion of the gene itself. While selecting the sequence for U′, care should be taken to account for the presence of a signal peptide. **b** In the case of C-terminal tags, the upstream sequence of the allele consists of a portion of the gene itself, while the downstream sequence (D′) consists of the region immediately downstream of the target gene. While selecting the sequence for D′, care must be taken to account for the presence of the endogenous stop codon. **c** In the case of gene replacements, U′ and D′ consist of sequences immediately upstream and downstream of the target gene respectively

Nikon, Japan). A 1.5× zoom lens was used for additional magnification on the camera. The NIS Elements software package (Nikon, Japan) was used for imaging. The 488 nm and 561 nm diode laser lines were applied sequentially to excite the eGFP and mCherry-tagged proteins respectively, and fluorescence emission was detected through a quad band filter (440/40, 521/21, 607/34, 700/45). Exposure time was set to 500 ms and 8× averaging was used to improve the signal-to-noise ratio. 16-bit images were recorded with an EM gain of 300 (without binning). The image size was 512 × 512 pixels, with a pixel size of 90 nm. Samples were maintained at ambient temperature during imaging. Images were analysed using Fiji (Schindelin et al. 2012).

Results

Construction of in-frame insertions (*atpG:egfp* and *smlt1054:egfp*)

To showcase the utility of this method in creating site-specific insertions, we chose to insert the eGFP-coding sequence in-frame of the ATP synthase γ subunit *atpG* (864 bp) and a phage-encoded endolysin *smlt1054* (486 bp) (Fig. 3). Based on experiments conducted in our laboratory, *atpG* associates with the inner cell membrane, while *smlt1054* is an endolysin, which is a part of the tailocin gene cluster that is induced in certain stress conditions such as exposure to fluoroquinolones (Devos et al. 2017).

Regardless of the type of insertion, there are some considerations of which to be mindful when designing the insert–

- If the insert is an entire gene (such as a fluorophore), it is important to consider the stop codon placement. Failure to account for this will result in insertion of the gene as its own stand-alone element instead of a fusion with the desired endogenous allele.
- If the insert is an entire gene, it is recommended to add a small linker (4–6 aa) in between the endogenous allele and the insert to improve stability of the resultant fusion protein. This linker can be designed to be flexible or rigid depending on one's requirements (Snapp 2005).
- Finally, if the endogenous gene is present on the minus-strand, the reverse-complement of the desired insert will have to be used.

In order to compare fusion protein stability and fluorescence output, we constructed both N- and C-terminal insertions for each gene; however, only C-terminal fusions were found to be fluorescent. Specific considerations for the construction of each type of insert are detailed below–

- *N-terminal insertions* The presence of a signal peptide should be accounted for– the insert should be designed such that it immediately follows the signal peptide sequence; otherwise it will be cleaved and will not be expressed. With regards to the placement of the stop codon– the stop codon from the *insert allele* should be omitted, so that the resulting fusion protein consists of the insert, a short linker, and the target gene with its endogenous stop codon.

- *C-terminal insertions* With regards to the placement of the stop codon– the stop codon from the *endogenous allele* should be omitted, so that the resulting fusion protein consists of the target gene, a short linker, and the insert allele.

To construct the insert allele for *atpG*, regions upstream (839 bp) and downstream (861 bp) of the gene were selected. The upstream region consisted of a portion of the gene immediately upstream (*atpD*), while the downstream region was comprised of the gene itself *without* its stop codon. Since *atpG* is found on the minus-strand, the reverse complements of the eGFP-coding sequence and linker were used. The final insert was made up of the upstream homology sequence (*atpD*), the eGFP and linker (coding for the amino acids GSGSGS) sequences, and the downstream homology sequence (*atpG*) (Supplementary Table S4). This sequence was synthesised and cloned into pGPI-SceI-XCm to give pAtpG-eGFP-C.

Similarly, to construct the insert allele for *smlt1054*, regions upstream (200 bp) and downstream (300 bp) of the gene were selected. The upstream region consisted of a portion of the gene immediately upstream (*smlt1053*), while the downstream region was comprised of a portion of the gene itself *without* its stop codon. *smlt1054* is also found on the minus-strand, hence the reverse complements of the eGFP-coding sequence and linker (coding for the amino acids GSGSGS) were used once again. The final insert was made up of the upstream homology sequence (*smlt1053*), the eGFP and linker sequences, and the downstream homology sequence (*smlt1055*) (Supplementary Table S4). This insertion only makes changes within the *smlt1054* ORF that is not involved in regulation of the maltocin operon. Therefore, it is not expected no to affect the induction of maltocin production and release (Sun et al. 2022). This sequence was synthesised and cloned into pGPI-SceI-XCm to give pSmlt1054-eGFP-C.

Suicide plasmids were transformed into recipient *S. maltophilia* through triparental mating using the helper plasmid pRK2013, and ex-conjugants were successfully obtained after 2 days. Following this, the endonuclease plasmid pDAI-SceI-SacB was introduced into these ex-conjugants, and transformants were seen after 2 days of incubation. Screening was performed by colony PCR using primers specific to the *egfp*-coding sequence and the target genes themselves (Supplementary Table S5)– in all, around 15–20 colonies (per mutation) needed to be screened to obtain the desired mutants. PCR fragments using gene-specific primers were sequenced to confirm correct insertion of eGFP (Fig. 4a and b). Multiple bands were observed when amplifying *smlt1054*, and this was also noted for several other primer pairs (data not shown). We concluded that the GC-rich nature of this region of the genome caused mispriming to occur, resulting in multiple bands. Sequencing of the largest band (around 2.5 kb) showed correct in-frame insertion of *egfp*.

Upon excitation with the 488 nm laser, eGFP fluorescence was observed for both mutants. For te h*atpG:egfp* mutant, fluorescence was observed at the boundaries of the cells (Fig. 4c, Supplementary Figure S1). However, we

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
DH5α	F^-, Φ80 *lacZ*ΔM15 (Δ*lacZYA-argF*)U169 *endA1 recA1 hsdR*17(rK^-mK^+) *supE*44 *thi*-1 Δ*gyrA*96 *relA*1	Lab collection (Grant et al. 1990)
HB101	F^-, *hsd*S20(r_B^- m_B^-) *supE*44 *leuB6 ara*14 *galK lac*Y1 *pro*A2 *rps*L20 *xyl5 mtl*1 *rec*A13	Boyer and Roulland-dussoix (1969)
SY327	F^-, Δ(lac pro) argE(am) rif malA recA56 λpir	Miller and Mekalanos (1988)
Stenotrophomonas maltophilia		
44/98	LMG 26,824, clinical isolate obtained from a patient in Italy (Mercuri et al. 2002)	BCCM/LMG
44/98 *atpG:egfp*	44/98, AtpG with C-terminal eGFP tag	This study
44/98 *smlt1054:egfp*	44/98, Smlt1054 with C-terminal eGFP tag	This study
44/98 *ax21:mCherry*	44/98, Ax21 with N-terminal mCherry tag, replacement of endogenous Ax21	This study
Plasmids		
pRK2013	ori_{colE1}, RK2 derivative, Kan^R, mob^+, tra^+	Figurski and Helinski (1979)
pGPI-SceI-XCm	ori_{R6K}, Tp^R, Cm^R, *traJ–I*, carries reporter protein XylE, carries I-SceI restriction site	Hamad et al. (2010)
pAtpG-eGFP-C	pGPI-SceI-XCm with *egfp* sequence flanked by *atpG* upstream and downstream homology regions	This study
pSmlt1054-eGFP-C	pGPI-SceI-XCm with *egfp* sequence flanked by *smlt1054* upstream and downstream homology regions	This study
pSmlt0387-mCherry-N	pGPI-SceI-XCm with N-terminal *smlt0387:mCherry* fusion protein construct	This study
pDAI-SceI-SacB	ori_{pBBR1}, Tc^R, carries counter-selectable marker SacB, carries I-SceI endonuclease-coding gene	Hamad et al. (2010)

BCCM/LMG– Belgian Coordinated Collection of Microorganisms/Laboratory of Microbiology, Ghent University

Kan– kanamycin, Tp– trimethoprim, Cm– chloramphenicol, Tc– tetracycline

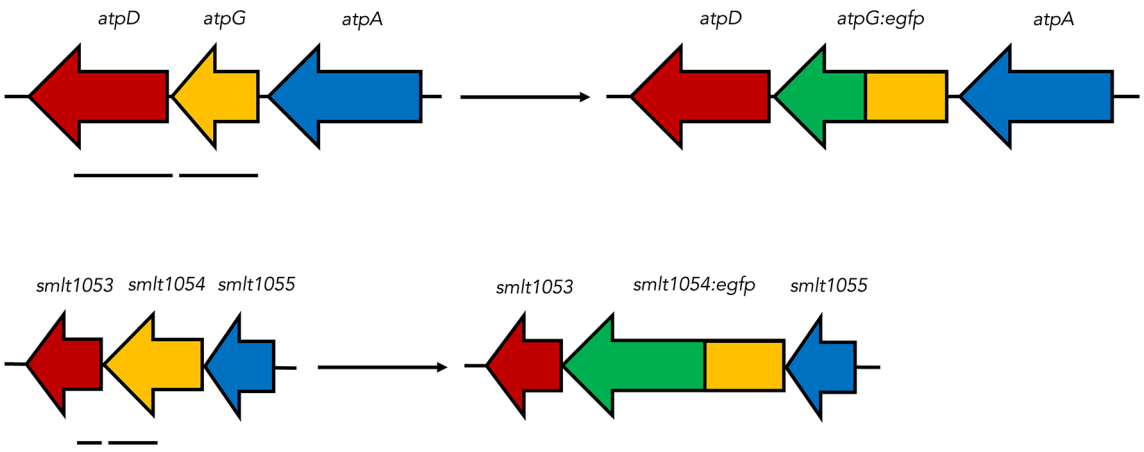

Fig. 3 Insertion schematic for*atpG: egfp* and *smlt1054:egfp*. (left) The upstream and downstream homology regions used for construction of the corresponding suicide plasmids are marked under each figure. (right) The resulting insertions (*atpG:egfp* and *smlt1054:egfp*) are visualised

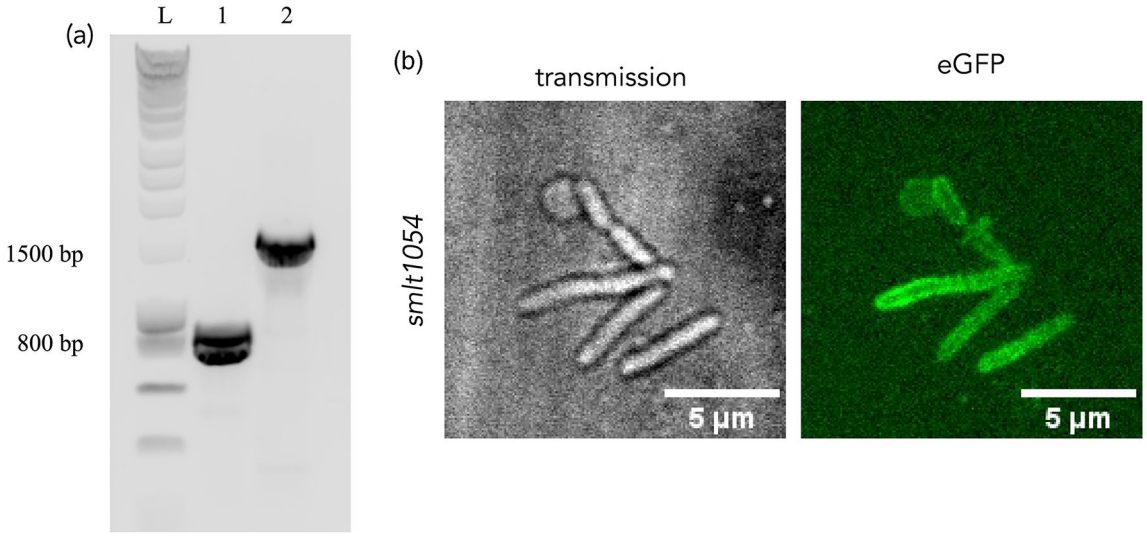

Fig. 4 Confirmation of*atpG:egfp* and *smlt1054:egfp* mutations. **a** PCR confirmation of*atpG:egfp* tag. L– ladder; 1– PCR of WT *atpG*; 2– PCR of *atpG: egfp*. **b** PCR confirmation of *smlt1054:egfp* tag. L– ladder; 1– PCR of eGFP insert (positive control); 2– PCR of WT *smlt1054*; 3– PCR of *smlt1054:egfp*. **c** Transmission (left) and fluorescence (right) microscopy images of *S. maltophilia* expressing *atpG:egfp*. **d** Transmission (left) and fluorescence (right) microscopy images of *S. maltophilia* expressing *smlt1054:egfp* upon exposure to 2 µg/ml norfloxacin

noticed that growth of this mutant was slightly impaired. Since the γ-subunit is an integral part of the bacterial F-type ATP synthase, we concluded that any attempt to interfere with this complex would result in poor growth. Induction of DNA stress upon addition of norfloxacin to the *smlt1054:egfp* mutant culture resulted in observable fluorescence for a subset of cells (Fig. 4d, Supplementary Fig. S1). Additionally, these cells were observed to be significantly longer than WT cells, a hallmark of cells in which the bacterial SOS response is activated. Fluorescence is only observed in the cells that are affected by the addition of norfloxacin and the sub-population of cells wherein spontaneous DNA damage is known to occur (Pennington and Rosenberg 2007; Turnbull et al. 2016).

Replacement of endogenous alleles (*smlt0387* with *smlt0387:mCherry*)

Finally, we aimed to show how our method could be used to replace an entire gene in a targeted manner. We selected to replace an outer membrane protein Smlt0387 (573 bp) with its N-terminal mCherry-tagged form Smlt0387:mCherry (1.3 kb) (Fig. 5). The role of this protein (named Ax21) is controversial, although it is believed to be implicated in virulence and biofilm formation (An and Tang 2018).

To construct the replacement allele, regions upstream (393 bp) and downstream (500 bp) of *smlt0387* were selected. The upstream region was comprised of mostly intergenic sequences and a small portion of the preceding gene (*smlt0386*), while the downstream region was made

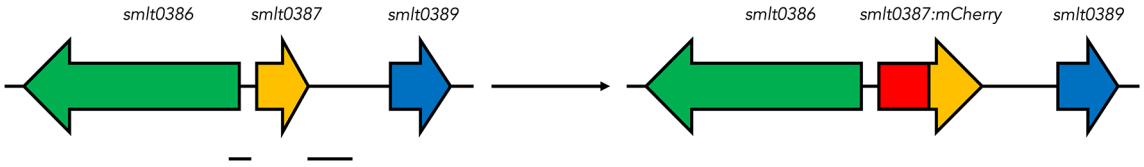

Fig. 5 Replacement schematic of *smlt0387*. (left) The upstream and downstream homology regions used for construction of the suicide plasmid are marked under the figure. (right) The resulting replacement (*smlt0387:mCherry*) is visualised

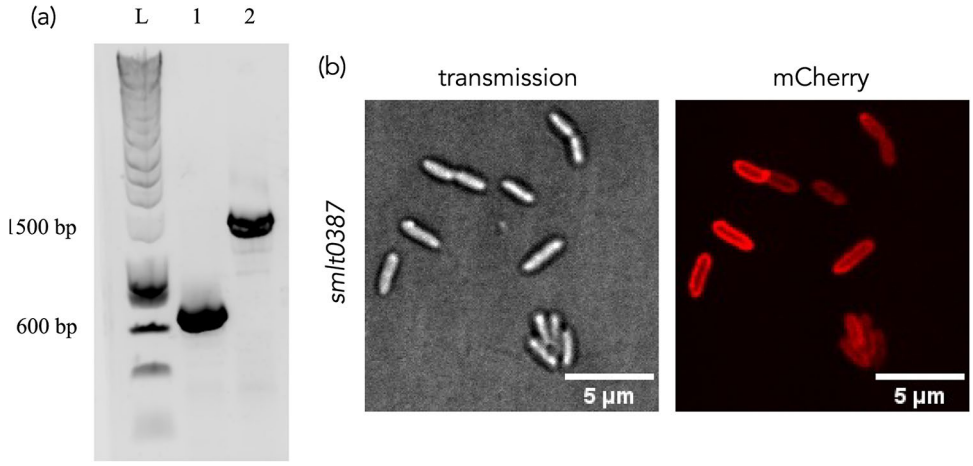

Fig. 6 Confirmation of *smlt0387:mCherry* replacement. **a** PCR confirmation of *smlt0387:mCherry* replacement. L– ladder; 1– PCR of WT *smlt0387*; 2– PCR of *smlt0387:mCherry*. **b** Transmission (left) and fluorescence (right) microscopy images of *S. maltophilia* expressing *smlt0387:mCherry*

up of entirely intergenic sequence. The replacement gene itself was comprised of the mCherry-coding sequence placed *after* the signal peptide sequence, followed by a short flexible linker (coding for the amino acid sequence GSGSGS) and the gene itself (Supplementary Table S4). The entire allele (2.5 kb) was synthesised and cloned into pGPI-SceI-XCm to give pSmlt0387-mCherry-N.

Once again, transformation of the suicide and endonuclease plasmids was performed as previously mentioned; however, the efficiency of the second transformation was noticeably lower (around 10 colonies per plate as compared to 20–40 for other types of mutations). Screening was performed through colony PCR using primers specific to *smlt0387*, where a small band was indicative of the WT allele and a large band was proof of its replacement (Supplementary Table S5). Ultimately, 20–25 colonies had to be screened from multiple plates to obtain the desired *smlt0387:mCherry* mutant– sequencing of the PCR fragment at the target site confirmed successful replacement (Fig. 6a). Upon excitation with the 561 nm laser, mCherry fluorescence localised to the cell periphery was easily observable (Fig. 6b, Supplementary Fig. S1).

Discussion

pGPI-SceI-XCm and design of mutant alleles

The pGPI-SceI-XCm suicide plasmid was constructed as an improved version of the pGPI-SceI plasmid for the purposes of mutagenesis in highly drug-resistant *B. cenocepacia* strains (Hamad et al. 2010). This plasmid contains the $ori_{R6K\gamma}$ which is dependent on the Pir protein for replication. This means that it can replicate in *E. coli pir*$^+$ strains but not in the target *S. maltophilia*, thus forcing the plasmid to integrate into the genome (under selective pressure) in order to be successfully propagated. As such, it is crucial to maintain this plasmid in an appropriate *pir*$^+$ strain of *E. coli*. In addition to all the elements from the original plasmid, it bears an additional chloramphenicol resistance cassette and the *xylE* reporter gene, which help in selection and screening of ex-conjugants respectively.

While designing the mutant allele, there are a few considerations to keep in mind–

1. The integration of pGPI-SceI-XCm into chromosomal DNA is mediated by HR. This integration is non-random due to the inclusion of appropriate homologous sequences, thereby eliminating the possibility of ectopic insertion. The likelihood of successful recombination increases with an increase in length of these homologous sequences, so they should be made long enough to facilitate this.
2. At the same time, the inherent (approximate) maximum 50% success rate of achieving the required mutation using this strategy compels one to make

adjustments elsewhere in order to maximise efficiency. We hypothesised that although the frequency of *plasmid integration* is dependent on the length of the upstream and downstream sequences, the frequency of *obtaining the required mutation* is directly dependent on the length of the downstream sequence. Therefore, we designed our inserts in such a way that the downstream homology sequence was longer than the upstream sequence, so as to bias recombination to occur downstream during repair of the DSB brought about by I-SceI. As such, we noted that only a small number of colonies (usually around 15–20) needed to be screened to obtain the desired mutation; however, a comparison between homology sequences of equal length needs to still be tested to verify our theory.

We noted success with homology sequences of as few as 200 bp (upstream) and 300 bp (downstream). However, these sequences should be made longer when dealing with larger open reading frames (ORFs). A good rule of thumb that has been suggested is to increase the length by around 50–100 bp for every additional 1 kb of ORF length (Hmelo et al. 2015).

By far, the construction of suicide plasmids is the most tedious part of this protocol. In the interest of saving time, we opted to outsource synthesis and cloning to an external company; however, if the user chooses to construct their plasmids by themselves, methods such as Gibson assembly are well-suited (Gibson et al. 2009). Additionally, it is important to make use of a high-fidelity polymerase with proofreading activity for amplification of homology regions and inserts. This helps minimise the introduction of deleterious point mutations that might hinder transformation and recombination efficiency or affect the inserted sequence. As such, it is also recommended to sequence the plasmids with the inserts after cloning to verify constructs.

Method of transformation– triparental mating (conjugation) vs. electroporation

Transformation protocols reported for *S. maltophilia* are few and far between, but most seem to make use of triparental mating. This makes use of 3 types of cells– (1) a donor strain containing the suicide vector to be transferred, (2) a helper strain containing a plasmid bearing mobilisation genes to facilitate the transfer of the suicide vector, and (3) a recipient strain which receives the suicide vector. Additionally, we also optimised a protocol for electroporation of plasmids.

For transformation of pGPI-SceI-XCm, we noted far fewer ex-conjugants (around 5–10 per plate) and far more false-positives (around 50%) when using electroporation when compared to triparental mating. This is not entirely surprising, since electroporation protocols have typically been well-optimised for model organisms and are far less efficient when working with other bacteria. In our experience, we noted an increase in transformation efficiency (defined as the number of true transformants to the total number of colonies obtained) when using polymyxin B nonapeptide (PMBN) during the initial growth phase of the cells. PMBN has been demonstrated to increase the electroporation efficiency in *Salmonella* by increasing uptake of genetic material, and we made a similar observation in *S. maltophilia* (Qin et al. 2022). For transformation of pDAI-SceI-SacB, we observed that electroporation yielded a comparable amount of screenable colonies (comprised of both true and false-positives) much quicker than triparental mating. This allows for quicker but more resource-intensive screening, if so desired.

We report both protocols here since all clinical isolates are different– the user can use our optimised protocols as a starting point for their experiments and accordingly choose the transformation method that works best.

Screening for transformants

The presence of the *xylE* reporter gene on pGPI-SceI-XCm greatly simplifies screening for transformants, even eliminating the need for PCR confirmation when screening for ex-conjugants. The product of this gene is catechol dioxygenase, which catalyses the conversion of pyrocatechol into a distinctively yellow pigment (hydroxymuconic semialdehyde) (Ingram et al. 1989).

After transformation of the suicide plasmid, colonies of sufficient size (>2 mm diameter) were sprayed using a 0.45 M solution of pyrocatechol. Transformants that turned yellow (usually in 30–40 s) were considered to be successful ex-conjugants. From our experience, colonies obtained at this stage are always true transformants due to sufficient selective pressure, since in theory, the plasmid will not be able to propagate over several generations without the Pir protein. However, it is still a good idea to confirm that the colony is in fact a recipient *S. maltophilia* as opposed to a (very unlikely) donor *E. coli*. Further, we recommend starting liquid cultures of at least 2 such ex-conjugants at this stage to prepare frozen stocks. These can be maintained until the desired mutation is achieved, providing an intermediate stopping point for subsequent transformation.

While screening transformants using PCR after transformation of pDAI-SceI-SacB, the type of primers used depends on the type of mutation–.

1. *For insertions* Primers specific to a small portion of the insert can be used to quickly screen for insertion– no band would be expected in the case of the negative control (WT DNA). Further

confirmation can be done using primers that flank the target gene or flank the insert site itself– in the case of the former, the appearance of large band relative to the positive control indicates successful insertion, while in the case of the latter, presence of a band would indicate successful insertion.

2. *For replacements* Similar to insertions, the user is free to design primers however they find convenient.

At this stage, the use of pyrocatechol to distinguish true transformants from false-positives has some drawbacks. Plates sprayed with pyrocatechol will turn dark brown/black rapidly due to exposure of the solution to oxygen, and cells may become unviable for further propagation. This can be solved in 2 ways– the user can spray plates with pyrocatechol and start liquid cultures of only true transformants (i.e., colonies that remain white), or the user can forgo the use of pyrocatechol altogether and perform colony PCR using a small portion of each colony. The former sacrifices time for more selectivity for time, while the opposite is true for the latter. In case the user decides to perform colony PCRs, it becomes necessary to verify that any potential interesting transformants are true transformants and not false-positives. This can be done using suicide plasmid-specific primers (no amplification would be indicative of excision of the integrated plasmid), or by re-streaking the corresponding colony on a fresh agar plate to spray with pyrocatechol (absence of the intense yellow colour would also indicate absence of the integrated plasmid).

One more point of concern for the reader might be the antibiotic resistance profiles of their MDR organism of interest and how to select for transformants if they show some intrinsic resistance to any of the antibiotics used on the plasmids in this study. In light of this, we recommend performing an initial minimum inhibitory concentration (MIC) experiment to determine the baseline level of resistance (if any) to all antibiotics used in this strategy. When implementing this method, we recommend using selection concentrations of up to 10 times the determined MICs as a starting point– if this is not enough to select for the appropriate plasmid, selection concentration can then be increased further. Failing this, an increase in the counter-selection antibiotic can also help eliminate donor and helper *E. coli* cells that might obscure true transformants. For example, in the case of strain 44/98, baseline resistance to Tc was observed at around 10 µg/ml and a selective concentration of 15–20 µg/ml was found to be selective enough when combined with increased counter-selection using Nor. In this way, even highly resistant organisms can be manipulated.

Comparison with other methods and applications of I-SceI-based mutagenesis

Allelic-exchange mutagenesis is not a new concept– indeed, it has been used for genetic manipulation of other Gram-negative bacteria, most notably in *P. aeruginosa* with pEX18-derived suicide plasmids (Hmelo et al. 2015). The I-SceI endonuclease strategy first optimised for use in *B. cenocepacia* has even been used for use in *S. maltophilia* to create unmarked deletions; however, these studies have been restricted to the type-strain (strain K279a) and only for deleting genes (Abda et al. 2015; Steinmann et al. 2018). Other studies have made use of replicative plasmids to transiently introduce fluorophores into environmental isolates (Li et al. 2019). More recently, a mini-Tn7 transposon-based delivery system was developed for *S. maltophilia*. The authors were successful in tagging a conserved intergenic site downstream of the *glmS* gene with different fluorophores; however, these tags were not associated with specific proteins, but aimed to label the cells as a whole (Mamat et al. 2023).

To the best of our knowledge, no studies thus far have reported site-specific introduction of insertions and/or gene replacements, least of all in non-model strains. As such, there is no single published protocol that demonstrates the creation of deletions, insertions, and gene replacements in atypical isolates using our strategy. To rectify this poorly-documented gap in knowledge, we have also included a table with common problems and troubleshooting steps as a guide to those eager to implement this method into their own research (Supplementary Table S6).

Using a multidrug-resistant clinical isolate of *S. maltophilia* as a proxy for non-classical Gram-negative bacteria, we present here a comprehensive account of our optimised strategy to tag genes with fluorescent proteins and replace an entire gene with a fluorophore-tagged form. Aside from this, we have created multiple combinations of deletions and insertions (including a deletion of *smlt1054* in an *ax21:mCherry* mutant and an *atpG:egfp ax21:mCherry* double-tagged mutant). Further, we also created multiple mutants which did not show any fluorescence (including a zinc uptake transporter *zupT* tagged with *egfp* at both termini). Other researchers may also find this strategy useful for the insertion of smaller tags (such as 6x His-tags for protein purification). We are confident that our efforts, combined with those of subsequent users, will pave the way for better understanding and application of other poorly-documented (highly-resistant) Gram-negative bacteria as well.

Abbreviations

DSB	Double strand break
eGFP	Enhanced green fluorescent protein
HR	Homologous recombination
LB	Lysogeny broth

MDR	Multidrug resistance
MIC	Minimal inhibitory concentration
ORF	Open reading frame
PCR	Polymerase chain reaction
PIA	*Pseudomonas* Isolation Agar
PMBN	Polymyxin B nonapeptide
WT	Wild type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13568-025-01844-2.

Supplementary Material 1

Acknowledgements
The authors express their gratitude to the Ghent University Light Microscopy (GLiM) CORE at Ghent University for the use and support on the spinning disk microscope.

Author contributions
DC conceptualised and carried out all experiments and wrote the manuscript. BD supervised the work, assisted in experiment conceptualisation, and reviewed manuscript.

Funding
was obtained from Fonds Wetenschappelijk Onderzoek (FWO) Grant Number G005719N and Belspo PRODEX as part of the Nanomotion project. This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability
Optimised transformation and screening protocols, primers used, microscopy images of successful mutants, and a troubleshooting guide for researchers can be found within the manuscript and the corresponding Supplementary Information.

Declarations

Competing interests
The authors state no relevant financial and/or non-financial competing interests.

Received: 23 January 2025 / Accepted: 12 February 2025
Published online: 28 February 2025

References

Abda EM, Krysciak D, Krohn-Molt I, Mamat U, Schmeisser C, Förstner KU, Schaible UE, Kohl TA, Nieman S, Streit WR (2015) Phenotypic heterogeneity affects *Stenotrophomonas maltophilia* K279a colony morphotypes and β-lactamase expression. Front Microbiol 6:1373

An S, Tang J (2018) The Ax21 protein influences virulence and biofilm formation in *Stenotrophomonas maltophilia*. Arch Microbiol 200:183–187. https://doi.org/10.1007/s00203-017-1433-7

Boyer HW, Roulland-dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Bio 41:459–472. https://doi.org/10.1016/0022-2836(69)90288-5

Crick F (1970) Central dogma of molecular biology. Nature 227:561–563. https://doi.org/10.1038/227561a0

Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream M-A, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB (2008) The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biol 9:R74. https://doi.org/10.1186/gb-2008-9-4-r74

Devos S, Stremersch S, Raemdonck K, Braeckmans K, Devreese B (2016) Intra- and interspecies effects of outer membrane vesicles from *Stenotrophomonas maltophilia* on β-lactam resistance. Antimicrob Agents Chemother 60:2516–2518. https://doi.org/10.1128/aac.02171-15

Devos S, Van Oudenhove L, Stremersch S, Van Putte W, De Rycke R, Van Driessche G, Vitse J, Raemdonck K, Devreese B (2015) The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. Front Microbiol. https://doi.org/10.3389/fmicb.2015.00298

Devos S, Van Putte W, Vitse J, Van Driessche G, Stremersch S, Van Den Broek W, Raemdonck K, Braeckmans K, Stahlberg H, Kudryashev M, Savvides SN, Devreese B (2017) Membrane vesicle secretion and prophage induction in multidrug-resistant *Stenotrophomonas maltophilia* in response to ciprofloxacin stress. Environ Microbiol 19:3930–3937. https://doi.org/10.1111/1462-2920.13793

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76:1648–1652. https://doi.org/10.1073/pnas.76.4.1648

Flannagan RS, Linn T, Valvano MA (2008) A system for the construction of targeted unmarked gene deletions in the genus Burkholderia: targeted unmarked gene deletions in *Burkholderia*. Environ Microbiol 10:1652–1660. https://doi.org/10.1111/j.1462-2920.2008.01576.x

Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318

Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc Natl Acad Sci USA 87:4645–4649. https://doi.org/10.1073/pnas.87.12.4645

Hamad MA, Skeldon AM, Valvano MA (2010) Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. Appl Environ Microbiol 76:3170–3176. https://doi.org/10.1128/AEM.03024-09

Hmelo LR, Borlee BR, Almblad H, Love ME, Randall TE, Tseng BS, Lin C, Irie Y, Storek KM, Yang JJ, Siehnel RJ, Howell PL, Singh PK, Tolker-Nielsen T, Parsek MR, Schweizer HP, Harrison JJ (2015) Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. Nat Protoc 10:1820–1841. https://doi.org/10.1038/nprot.2015.115

Ingram C, Brawner M, Youngman P, Westpheling J (1989) xylE functions as an efficient reporter gene in *Streptomyces* spp.: use for the study of galP1, a catabolite-controlled promoter. J Bacteriol 171:6617–6624

Kendrew JC, Bodo G, Dintzis HM, Parrish RG, Wyckoff H, Phillips DC (1958) A three-dimensional model of the myoglobin molecule obtained by X-ray analysis. Nat 181:662–666. https://doi.org/10.1038/181662a0

Kolisnychenko V, Plunkett G, Herring CD, Fehér T, Pósfai J, Blattner FR, Pósfai G (2002) Engineering a reduced *Escherichia coli* genome. Genome Res 12:640–647. https://doi.org/10.1101/gr.217202

Li Y-L, Zheng C-Y, Liu K-C, Wu Y, Fan B, Han Z-M (2019) Transformation of multi-antibiotic resistant *Stenotrophomonas maltophilia* with GFP gene to enable tracking its survival on pine trees. For 10:231. https://doi.org/10.3390/f10030231

Mamat U, Hein M, Grella D, Taylor CS, Scholzen T, Alio I, Streit WR, Huedo P, Coves X, Conchillo-Solé O, Gómez A-C, Gibert I, Yero D, Schaible UE (2023) Improved mini-Tn7 delivery plasmids for fluorescent labeling of *Stenotrophomonas maltophilia*. Appl Environ Microbiol 89:e00317-e323. https://doi.org/10.1128/aem.00317-23

Mercuri PS, Ishii Y, Ma L, Rossolini GM, Luzzaro F, Amicosante G, Franceschini N, Frere J-M, Galleni M (2002) Clonal diversity and metallo-β-lactamase production in clinical isolates of *Stenotrophomonas maltophilia*. Microb Drug Resist 8:193–200. https://doi.org/10.1089/107662902760326904

Miller VL, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J Bacteriol 170:2575–2583. https://doi.org/10.1128/jb.170.6.2575-2583.1988

Muirhead H, Perutz MF (1963) Structure of haemoglobin: a three-dimensional Fourier synthesis of reduced human haemoglobin at 5.5 Å resolution. Nat 199:633–638. https://doi.org/10.1038/199633a0

Pauling L, Corey RB (1951) Atomic coordinates and structure factors for two helical configurations of polypeptide chains. Proc Natl Acad Sci USA 37:235–240. https://doi.org/10.1073/pnas.37.5.235

Pennington JM, Rosenberg SM (2007) Spontaneous DNA breakage in single living *Escherichia coli* cells. Nat Genet 39:797–802. https://doi.org/10.1038/ng2051

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229–233. https://doi.org/10.1016/0378-1119(92)90691-H

Qin J, Hong Y, Pullela K, Morona R, Henderson IR, Totsika M (2022) A method for increasing electroporation competence of Gram-negative clinical isolates by polymyxin B nonapeptide. Sci Rep 12:11629. https://doi.org/10.1038/s41598-022-15997-8

Rudner DZ, Losick R (2010) Protein subcellular localization in bacteria. Cold Spring Harb Perspect Biol 2:a000307. https://doi.org/10.1101/cshperspect.a000307

Sanger F (1949) The terminal peptides of insulin. Biochem J 45:563. https://doi.org/10.1042/bj0450563

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. https://doi.org/10.1038/nmeth.2019

Sharan SK, Thomason LC, Kuznetsov SG, Court DL (2009) Recombineering: a homologous recombination-based method of genetic engineering. Nat Protoc 4:206–223. https://doi.org/10.1038/nprot.2008.227

Snapp E (2005) Design and use of fluorescent fusion proteins in cell biology. Curr Protoc Cell Biol 27(1):21.4.1-21.4.13. https://doi.org/10.1002/0471143030.cb2104s27

Steinmann J, Mamat U, Abda EM, Kirchhoff L, Streit WR, Schaible UE, Niemann S, Kohl TA (2018) Analysis of phylogenetic variation of *Stenotrophomonas maltophilia* reveals human-specific branches. Front Microbiol. https://doi.org/10.3389/fmicb.2018.00806

Sumner JB (1926) The isolation and crystallisation of the enzyme urease. J Biol Chem 69:435–441. https://doi.org/10.1016/S0021-9258(18)84560-4

Sun W, Liang X, Zhu C, Xu Y, Ding Y, Huang Y-P (2022) Regulation of maltocin synthesis in *Stenotrophomonas maltophilia* by positive and negative regulators. Res Microb 173:103956. https://doi.org/10.1016/j.resmic.2022.103956

Tischer BK, Smith GA, Osterrieder N (2010) En passant mutagenesis: a two step markerless red recombination system. In: Braman J (ed) In vitro mutagenesis protocols, 3rd edn. Humana Press, Totowa, pp 421–430

Turnbull L, Toyofuku M, Hynen AM, Kurosawa M, Pessi G, Petty NK, Osvath SR, Carcamo-Oyarce G, Gloag ES, Shimoni R, Omasits U, Ito S, Yap X, Monahan LG, Cavaliere R, Ahrens CH, Charles IG, Nomura N, Eberl L, Whitchurch CB (2016) Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nat Commun. https://doi.org/10.1038/ncomms11220

Van Oudenhove L, De Vriendt K, Van Beeumen J, Mercuri PS, Devreese B (2012) Differential proteomic analysis of the response of *Stenotrophomonas maltophilia* to imipenem. Appl Microbiol Biotechnol 95:717–733. https://doi.org/10.1007/s00253-012-4167-0

Yao R, Liu D, Jia X, Zheng Y, Liu W, Xiao Y (2018) CRISPR-Cas9/Cas12a biotechnology and application in bacteria. Synth Sys Biotechnol 3:135–149. https://doi.org/10.1016/j.synbio.2018.09.004

Zhang N, Shao L, Jiang Y, Gu Y, Li Q, Liu J, Jiang W, Yang S (2015) I-SceI-mediated scarless gene modification via allelic exchange in *Clostridium*. J Microbiol Methods 108:49–60. https://doi.org/10.1016/j.mimet.2014.11.004

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