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Cre-*lox*-Based Method for Generation of Large Deletions within the Genomic Magnetosome Island of *Magnetospirillum gryphiswaldense*▽†

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Magnetosome biomineralization and magnetotaxis in magnetotactic bacteria are controlled by numerous, mostly unknown gene functions that are predominantly encoded by several operons located within the genomic magnetosome island (MAI). Genetic analysis of magnetotactic bacteria has remained difficult and requires the development of novel tools. We established a Cre-*lox*-based deletion method which allows the excision of large genomic fragments in *Magnetospirillum gryphiswaldense*. Two conjugative suicide plasmids harboring *lox* sites that flanked the target region were subsequently inserted into the chromosome by homologous recombination, requiring only one single-crossover event, respectively, and resulting in a double cointegrate. Excision of the targeted chromosomal segment that included the inserted plasmids and their resistance markers was induced by *trans* expression of Cre recombinase, which leaves behind a scar of only a single *loxP* site. The Cre helper plasmid was then cured from the deletant strain by relief of antibiotic selection. We have used this method for the deletion of 16.3-kb, 61-kb, and 67.3-kb fragments from the genomic MAI, either in a single round or in subsequent rounds of deletion, covering a region of approximately 87 kb that comprises the *mamAB*, *mms6*, and *mamGFDC* operons. As expected, all mutants were Mag$^-$ and some were Mot$^-$; otherwise, they showed normal growth patterns, which indicates that the deleted region is not essential for viability in the laboratory. The method will facilitate future functional analysis of magnetosome genes and also can be utilized for large-scale genome engineering in magnetotactic bacteria.

Magnetosomes are unique membrane-enveloped organelles that are formed by magnetotactic bacteria (MTB) for magnetic navigation (2, 37). The mechanism of magnetosome formation is within the focus of a multidisciplinary interest and has relevance for biotechnological applications (5). It has been recognized that the biomineralization of inorganic magnetite crystals and their assembly into highly ordered magnetosome chains are under strict genetic control. Recent studies combining proteomic and bioinformatic approaches suggested that the genetic determination of magnetosome formation is complex and may potentially involve 25 to 50 gene functions (15), with unknown numbers of accessory genes and those controlling signal transduction and motility to achieve effective magnetotaxis (8, 9, 12, 26, 27, 29). However, the functional characterization of these candidate genes has been lagging behind. This is due to technical difficulties and the lack of facile tools for genetic manipulation of MTB. Allelic replacement systems have been established for *Magnetospirillum magneticum* (18) and *Magnetospirillum gryphiswaldense* (39, 40), but so far, there are only few examples of these for magnetosome genes that were functionally characterized because of the tedious and cumbersome procedures required for mutant generation (11, 19, 28, 31–32). Most genes controlling magnetosome formation in these and other MTB are located within a genomic magnetosome island (MAI) (34), which is genetically instable during stationary growth (47) and more or less conserved in other MTB (12, 13, 35). Most known magnetosome genes are organized within several conserved operons, which are interspersed with large, poorly conserved genome sections of unknown functions that have been speculated to represent genetic junk irrelevant for magnetotaxis but to cause genetic instability by their high content of repeats and transposable elements (34, 47). Thus, for large-scale functional genome analysis and rearrangements of the MAI, there is a great need for additional and more efficient genetic methods.

Artificial genome recombination systems have been described for a number of bacteria. Many of them are based on the Cre-*loxP* system of the P1 phage (42). The Cre-*loxP* recombination system is a simple two-component system that is recognized as a powerful genetic tool in a multitude of eukaryotic and prokaryotic organisms (4, 6, 48). The Cre protein belongs to the integrase family of site-specific recombinases and catalyzes reciprocal site-specific recombination of DNA at 34-bp *loxP* sites, resulting in either excision or inversion, depending on the parallel or antiparallel orientation of the *loxP* sites, respectively (21). It does not require any host cofactors or accessory proteins (7). Cre-*lox* deletion has several advantages over other methods, such as a high efficiency and the independency of the length of DNA located between the two *lox* sites. The utility of Cre-*lox* systems has been demonstrated in a wide variety of Gram-positive and Gram-negative bacteria (17, 22–23). In several studies, it was applied for the generation of large-scale deletions, as in for example, the Gram-positive *Corynebacterium glutamicum* (43–46) and *Bacillus subtilis* (49).

In *M. gryphiswaldense*, the functionality of a Cre-*loxP* anti-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference(s) or source
M. gryphiswaldense strains		
MSR-1 R3/S1	Rif^r Sm^r, spontaneous mutant	40
MSR-1B	Spontaneous mutant, lacking 40.385 kb genomic region	34 and 47
MSR-1_SU1	MSR-1::pSU12	This study
MSR-1_SU4	MSR-1::pSU12::pSU13	This study
Δ*mamAB*#K7	Δ*mamAB*	This study
Δ*mamAB*#K7_pSU28	Δ*mamAB*::pSU28	This study
MSR-1_SU12	Δ*mamA*B with deletion to *mgr4029*	This study
MSR-1B_SU13	MSR-1B::pSU25	This study
MSR-1B_SU14	MSR-1B::pSU25::pSU37	This study
MSR-1BΔ*mgr4058*to*mgr4146*	MSR-1B extension of deletion from *mgr4058* to *mgr4146*	This study
E. coli strain BW29427	*dap* auxotroph derivative of *E. coli* strain B2155	B. Wanner
Plasmids		
pJet1.2	Ap^r, *eco47IR* (lethal restriction enzyme gene), *rep* (pMB-1)	Fermentas
pGEM-T Easy	Ap^r, *lacZ*α, PCR cloning vector	Promega
pKmob*GII*	Kn^r, pMB-1 replicon, *gusA*, *lacZ*α	16
pAS200	Gm^r, COLE1 ori, *sacB* of *Bacillus subtilis*	This study
pCM184	Km^r Ap^r Tc^r	25
pCM157	Tc^r, Cre expression vector	25
pSU12	pKmob*GII* with upstream *mamAB*-flanking sequence in EcoRI	This study
pSU13	pAS200 with downstream *mamAB*-flanking region	This study
pSU25	pSU12 cut with XbaI and HincII, blunted, and ligated with upstream sequence of *mgr4058*	This study
pSU37	pCM184 cut with BglII and BsaXI, blunted, self-ligated, then cut with SalI and NheI, blunted, and self-ligated, resulted in pCM184 derivative with downstream *mgr4146*-flanking sequence in AgeI	This study
pSU28	pSU12 cut with XbaI and HincII and ligated sticky and blunt end with upstream sequence of *mgr4029*	This study

biotic marker recycling system (25) has been previously demonstrated by deletion of a single gene based on double-crossover insertion of two *loxP* sites, followed by subsequent Cre-mediated excision (31). In this study, we describe a novel strategy for Cre-*loxP*-mediated deletion of large genomic fragments which requires only two single crossovers. The system has been validated by the generation of three large deletions, two single and one combination within the MAI, which demonstrated that the total deleted region of approximately 87 kb is not essential for viability and growth in the laboratory.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains used in this study are presented in Table 1. Liquid cultures of all *Magnetospirillum* strains were grown in modified flask standard medium (FSM) (10). Colonies of *M. gryphiswaldense* were obtained on activated charcoal agar medium (ACAM) that was incubated at 30°C (41). For growth of *Escherichia coli* strains, lysogeny broth (3) was supplemented with 1 mM DL-α,ε-diaminopimelic acid (Sigma-Aldrich, Switzerland). Culture conditions for *E. coli* strains were as previously described (30). Antibiotics were used at the following concentrations for *E. coli*: 25 μg/ml kanamycin (Km), 12 μg/ml tetracycline (Tet), and 15 μg/ml gentamicin (Gm). Antibiotics were used at the following concentrations for *M. gryphiswaldense* strains: 5 μg/ml kanamycin, 5 μg/ml tetracycline, and 20 μg/ml gentamicin. The pKmob*GII* plasmid contains a chromogenic marker, the *gusA* gene, which encodes the β-glucoronidase (GUS) enzyme. The concentration of the substrate X-Gluc (5-bromo-4-chloro-3-indoxyl-β-D-glucuronidase) (AppliChem GmbH, Germany) was 50 μg/ml.

The optical density and magnetic response (C_{mag}) of *M. gryphiswaldense* R3/S1 cultures were measured turbidimetrically at 565 nm as previously described (38). For conjugation experiments, *E. coli* strain BW29427 was used as a donor and cultivated (K. Datsenko and B. L. Wanner, unpublished data) as previously described (33). Conjugative transfers of plasmids were performed as described by Schultheiss et al. (40), with slight modifications. For selection of homologous recombination events, up to 5×10^9 cells were mixed and incubated microaerobically on ACAM for 8 h. Cells were flushed from the agar surface into sterile medium. To increase the ratio of homologous recombination events, the cells were incubated in this medium for 2 h before they were plated onto ACAM with the appropriate antibiotics for plasmid selection.

DNA techniques. Total DNA from *M. gryphiswaldense* strains used in this study was isolated as described previously (24). Genetic constructs used in this work were generated using standard PCR procedures. Primer sequences for amplification of DNA fragments from *M. gryphiswaldense* MSR-1 were derived from GenBank sequence deposition no. CU459003. For sequencing, we used BigDye Terminator version 3.1 chemistry on an ABI 3700 capillary sequencer (Applied Biosystems, Darmstadt, Germany). Sequence data were analyzed with Lasergene 6 (DNAStar Inc., Madison, WI). Primers were purchased from Sigma-Aldrich (Steinheim, Germany) and MWG Biotech (Ebersberg, Germany). For Southern blot hybridization, DNA was isolated, digested with restriction enzymes, electrophoresed, and blotted on a Hybond-N membrane (Amersham). Probe DNA was labeled with radioactive [α-^{32}P]dATP by using the HexaLabel kit (Fermentas, St. Leon-Rot, Germany) and the primers SondemamABfw and SondemamABrw. Prehybridization and hybridization were carried out at 65°C. Signals were detected with a PhosphorImager Typhoon 9400 scanner (Amersham Pharmacia).

Construction of the *loxP* site plasmids. For the construction of the inserted *loxP* plasmids we amplified flanked regions upstream and downstream of the deletion targets. The PCR products were cloned into pGEM-T Easy or pJET1.2 plasmids, sequenced, and subcloned into suicide plasmids. For the construction of the *mamAB* deletion mutant, a 1.453-kb fragment was amplified by PCR by using primers 5′mamABfw_SU and 5′mamAB_SUloxP (see Table S1 in the supplemental material), which included the *loxP* sequence, and then subcloned into the pGEM-T Easy vector (Promega, Mannheim, Germany). The upstream fragment of the *mamAB* operon was sequenced and excised with EcoRI and ligated with the pKmob*GII* vector containing the *gusA* gene (16), resulting in pSU12. pKmob*GII* carries, besides kanamycin resistance, the chromogenic *gusA* marker. The downstream construct of the *mamAB* operon was PCR amplified using the primers 3′mamABfw_SUloxP and 3′mamABrw_SU. The resulting 953-bp fragment was sequenced and subcloned into pAS200 (constructed from pBBRMCS5 by replacing *oriV* with the *COLE1* origin of replication [ori] and

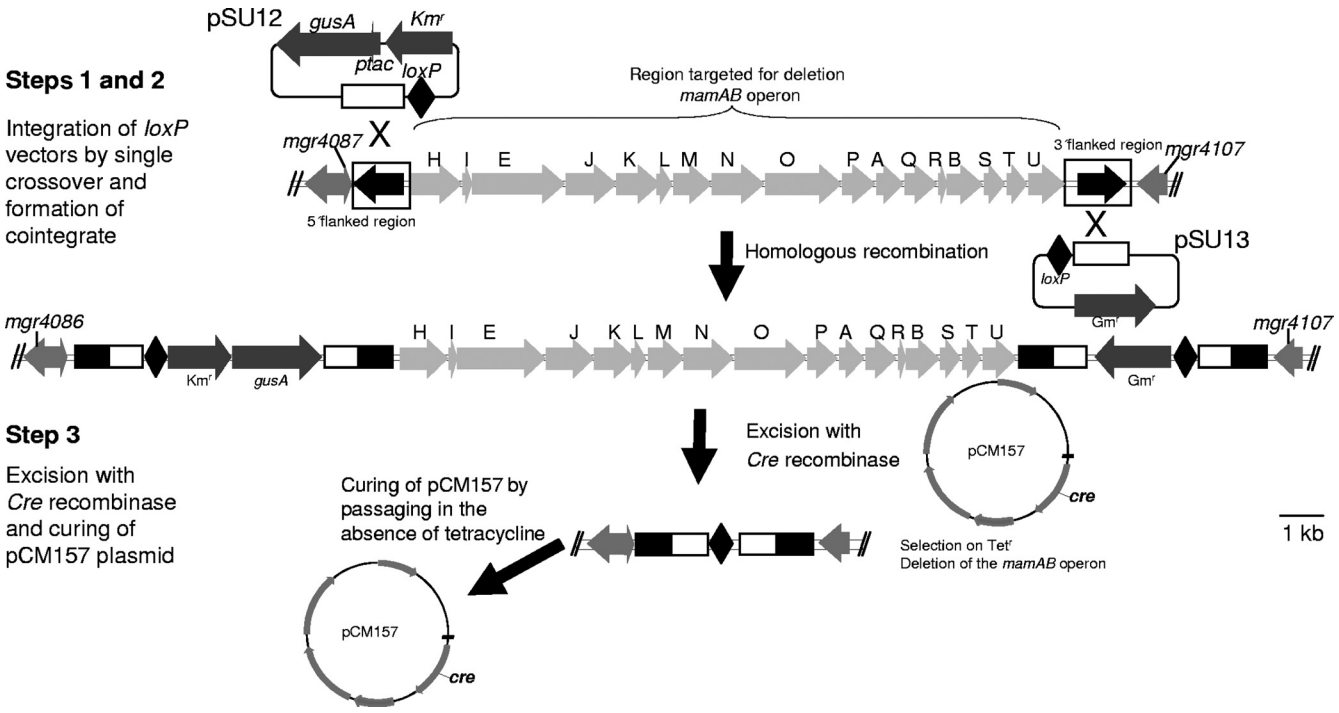

FIG. 1. Schematic representation of steps applied for the generation of a Cre-*loxP*-mediated 16.3-kb genomic deletion within the genomic magnetosome island (MAI) comprising the entire *mamAB* operon. Other deletions were generated in an analogous way. See the text for details.

inserting a *sacB* gene) to yield pSU13. pSU12 was cut with XbaI and HincII to delete the 5′-end *mamAB* fragment without the *loxP* site. pKmob*GII* containing one *loxP* site was used for cloning of further upstream fragments. pSU25 was generated from a 2,539-bp fragment localized 345 bp from the spontaneous deletion of the MSR-1B strain and cloned sticky and blunt into pKmob*GII*::*loxP* using XbaI and HincII. The same cloning steps were used to create the pSU28 plasmid containing a 2,391-bp sequenced fragment. pSU37 contained a downstream fragment of *mgr4146* of 2,337 bp, localized approximately 27 kb downstream of the spontaneous deletion. This fragment was amplified by PCR by using primers 3′MGR4146SUfw and 3′MGR4146SUrw and then subcloned into pGEM-T Easy vector. The fragment was excised with NotI, blunted, and ligated blunt end into the AgeI cleavage site of a pCM184 derivate containing one *loxP* site and a gentamicin cassette. The gentamicin cassette was subcloned blunt from the pBBRMCS5 plasmid with PstI.

RESULTS

Our strategy for the construction of large deletions relies on a combination of homologous and site-specific recombination, with the latter mediated by the Cre-*lox* system (Fig. 1). First, homologous regions (1 to 2.5 kb) flanking the 5′ and 3′ ends of the targeted regions, respectively, are cloned while each is adjacent to *loxP* sites on two different suicide vectors marked differentially by a kanamycin and a gentamicin resistance cassette, respectively. The vectors are then conjugated into the recipient and inserted via homologous recombination by single crossovers into the recipient chromosomes to form a double cointegrate. The relative orientations of the *lox* sites with respect to the homologous fragments are chosen such that they flank as direct repeats the entire target region encompassing the two inserted plasmids in the double cointegrate to allow subsequent excision of the vector sequences, including the resistance markers, leaving behind only a scar of one 34-bp *loxP* site. Thus, chromosome integration requires only two single crossovers, which are easier to select for than double crossovers that occur at low frequencies and have remained tedious to screen for in *M. gryphiswaldense* (14, 40). The efficiencies of single insertions were between 1.34×10^{-5} and 1×10^{-8}. Excision is then induced by conjugational transfer of the replicative plasmid pCM157 (25), from which the Cre recombinase is expressed in *trans*, resulting in the deletion of the targeted chromosomal segment (Fig. 1). The helper plasmid pCM157 is then cured from the deletant strain by relief of antibiotic selection, which had previously been observed to occur at a frequency of 10^{-1} after one transfer (31).

We first tested this strategy to delete the *mamAB* operon that was already known to be not essential for growth (34, 47) but was expected to have a nonmagnetic phenotype (Mag^-), as shorter spontaneous deletions mapping within the *mamAB* operon resulted in cells lacking magnetite crystals (47). The *mamAB* operon is localized within the MAI, extends over 16.4 kb, and comprises 17 genes of mostly unknown function that are cotranscribed from a single promoter (36). In addition to genes implicated in magnetite biomineralization, genes with functions in magnetosome chain assembly (*mamK* and *mamJ*) are carried within the operon (18, 32). For the generation of a defined 16.362-kb *mamAB* deletion, flanking homologous regions of 1.460 kb upstream and 0.952 kb downstream were cloned into the *lox* destination vectors derived from pKmob*GII* containing a *gusA* marker gene (16), resulting in pSU12 and pSU13, respectively. Conjugation of pSU12 yielded 54 Km-resistant colonies that were blue on X-Gluc plates due to the presence of β-glucuronidase encoded by *gusA*. After verification of proper chromosomal insertion in all clones by PCR (data not shown), one single-insertant clone named MSR-

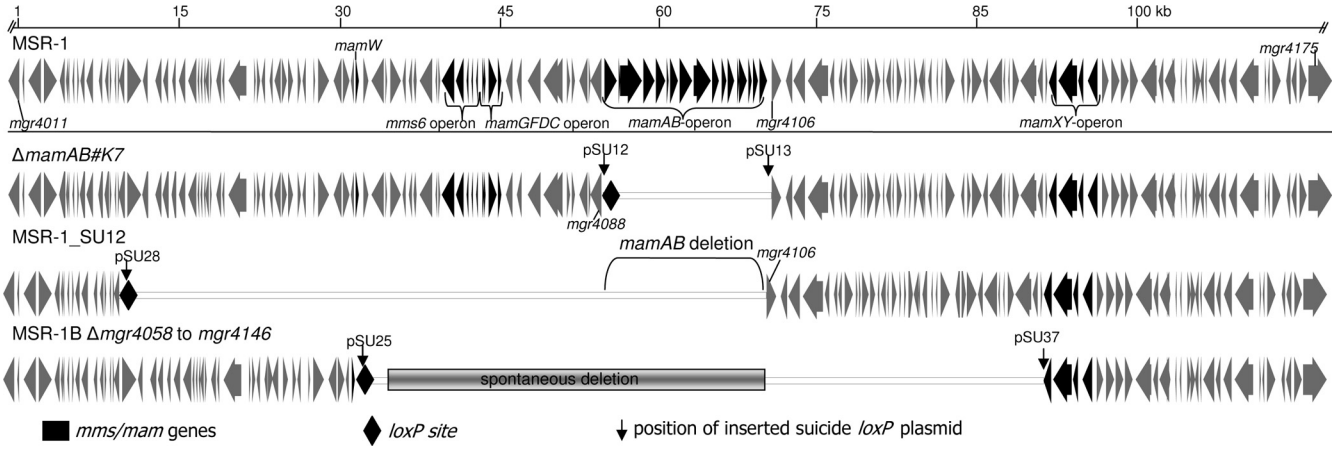

FIG. 2. Molecular organization of the MAIs of mutant strains after Cre-*loxP* deletion of three different genomic sections.

1_SU1 was selected. Subsequent conjugation of pSU13, which was derived from a pBBRMCS-5 backbone (20) by elimination of the origin of replication, into the single-insertant strain MSR-1_SU1 resulted in 10^{-6} to 1.2×10^{-8} double cointegrants (MSR-1_SU4) per recipient that were resistant to both kanamycin and gentamicin and formed blue colonies (16) (see Fig. S1A in the supplemental material). Growth and magnetite formation were not affected in MSR-1_SU4.

Lox-mediated excision of the targeted region was initiated by conjugational introduction of pCM157 carrying the Cre recombinase into double-cointegrate strain MSR-1_SU4. After several transfers, we used replica plating to identify 10 out of approximately 300 clones, sensitive to both kanamycin and gentamicin, that had lost their blue color due to loss of the plasmid-borne *gusA* gene, and hence had lost the targeted chromosomal segment (see Fig. S1B in the supplemental material). Precise excision was verified by PCR amplification and sequencing of a 1.5-kb fragment spanning the excision site and Southern blotting (see Fig. S1C in the supplemental material). To cure pCM157 from this strain, which was named Δ*mamAB*#K7, it was serially transferred in the absence of tetracycline. Clones that had lost the plasmid were identified after five consecutive passages by replica plating. Light microscopy, transmission electron microscopy (TEM), and C_{mag} results revealed that strain Δ*mamAB*#K7 had lost its capability to align in magnetic fields and to form magnetite crystals (Mag^-), whereas morphology, growth, and motility were unaffected, confirming that the *mamAB* operon is essential for magnetosome formation but is not required for growth.

Generation of multiple deletions. In the next experiments, we wanted to use the technique for genome engineering using the generation of multiple large deletions within the MAI. The target regions were selected because they were predicted to be involved in magnetosome synthesis but not essential for growth (47). As the Cre-mediated excision of *mamAB* left behind an intact *loxP* site in strain Δ*mamAB*#K7, we reused this site for another round of deletion (Fig. 2). Therefore, pSU28 harboring a second *loxP* site and a 2.5-kb fragment that is homologous to a chromosomal region 45 kb upstream of the left boundary of the *mamAB* operon was recombined via conjugation into deletion strain Δ*mamAB*#K7. Upon introduction of pCM157 harboring *cre*, the targeted fragment of 45 kb was precisely excised, as confirmed by PCR analysis, yielding strain MSR-1_SU12. Thus, a contiguous chromosomal region of 61 kb was eliminated in total by the two consecutive rounds of deletion. Like Δ*mamAB*#K7, strain MSR-1_SU12 displayed no phenotype other than loss of magnetosomes (Mag^-); that is, its morphology, growth characteristics, and motility were indistinguishable from those of the wild type.

In a further example, we used strain MSR-1B as a parent, which is Mag^- due to a spontaneous deletion of 40 kb comprising a part of the MAI (34, 47). We wanted to further extend this deletion by excision of an additional 27-kb fragment adjacent to the deletion site (Fig. 2). Homologous fragments flanking the targets of 2.539 and 2.337 kb were cloned into *lox*-pKmob*GII* and a pCM184 derivate, yielding pSU25 and pSU37, respectively. Consecutive insertion and subsequent Cre excision yielded 4 out of 26 Tet^r Kan^s Gen^s clones in which the target region was properly excised, as verified by PCR, resulting in strain MSR-1BΔ*mgr4058*to*mgr4146*. In total, the extent of the chromosomal deletion in strain MSR-1BΔ*mgr4058*to*mgr4146* was 67.345 kb. Strain MSR-1BΔ*mgr4058*to*mgr4146* showed a phenotype identical to that of its parental strain MSR-1B, i.e., it was Mag^- and Mot^- (nonmotile) but displayed otherwise normal growth and morphology.

DISCUSSION

We describe a strategy for the generation of large-scale deletions by site-specific recombination in magnetotactic bacteria. Compared to conventional deletion, the Cre-*loxP*-based method provides several advantages. First, it is very efficient on large fragments, and marker recycling by the site-specific Cre recombinase enables the construction of strains bearing multiple genetic modifications. We have also demonstrated that the single *loxP* site that remains inserted can be recycled by reusing it in consecutive rounds of deletion. For example, by the combination of a single *loxP* site with different *loxP* insertions, the generation of a range of genome deletions with variable lengths has become straightforward. Similar methods were first described for use in *E. coli* by vector-mediated excisions (VEX) (1) and later applied during genome engineering

in *C. glutamicum* (46). Our method has been specifically adapted for application in magnetospirilla by the modification of a pair of conjugative suicide vectors for *lox* introduction. In addition, the presence of the *gusA* gene (16) encoding β-glucoronidase on one of the vectors allows it to monitor vector insertion and subsequent excision. Another specific advantage of our strategy is that it solely relies on single crossovers, which are easier to enforce than double-crossover events (31, 40). Frequencies of RecA-mediated single-crossover insertions in *M. gryphiswaldense* were previously reported to be 10^{-6} compared to frequencies of 10^{-8} for double crossovers (40). In the presented examples and further unpublished mutagenesis experiments, we observed single insertion frequencies between 2×10^{-3} and 1×10^{-6}. Subsequent Cre-mediated deletion of the target fragment could usually be detected at approximately 10^{-3} after 3 to 7 passages. This was significantly less frequent compared to a frequency of 1.0×10^{-1} after only one passage, as previously described (31), and seemed to vary in an unpredictable manner depending on the particular target and the distance between the *lox* sites. Precise excision of the target fragments was found in about 4 to 15% of the tested Kan^s Gen^s $GusA^-$ clones. The total time requirement to generate a mutant using this method depended on the particular region, but generation could be usually achieved within several weeks. This is generally faster and more efficient than using conventional allelic replacement techniques for construction of unmarked deletion methods.

We have demonstrated the usefulness of this method for the construction of 16.3-kb, 61-kb, and 67.3-kb deletions within the genomic MAI, either alone or in combination. In total, a region of approximately 87 kb was covered by overlapping deletions, comprising the *mamAB*, *mms6*, and *mamGFDC* operons. As was expected from previous genetic analysis (47), all mutants were nonmagnetic (Mag^-) and some were nonmotile (Mot^-), but otherwise they showed normal growth patterns under the tested conditions. This indicates that the deleted regions are not essential for viability in the laboratory, which further corroborates our previous assumption that the regions neighboring the known magnetosome operons within the MAI rather comprise accessory genes that are dispensable depending on environmental conditions and might be lost or acquired horizontally (12).

As vectors and techniques are compatible with the closely related magnetotactic bacterium *M. magneticum*, the described strategy probably can also be used in the latter strain and potentially other MTB. It can be further envisioned that this method can be used for several applications in future approaches. In addition to a systematic functional analysis of the genomic MAI, it could be utilized for targeted genome engineering in magnetotactic bacteria. For example, genomic regions which are not required for growth and magnetosome formation but which contain a large number of repeats and transposons that cause instability of the MAI could be eliminated. This would result in genetically stable host strains synthesizing magnetosomes, which would facilitate the production and application of the biogenic magnetic nanoparticles in biotechnology.

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