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Genome Editing Method for the Anaerobic Magnetotactic Bacterium *Desulfovibrio magneticus* RS-1

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ABSTRACT Magnetosomes are complex bacterial organelles that serve as model systems for studying bacterial cell biology, biomineralization, and global iron cycling. Magnetosome biogenesis is primarily studied in two closely related *Alphaproteobacteria* of the genus *Magnetospirillum* that form cubooctahedral-shaped magnetite crystals within a lipid membrane. However, chemically and structurally distinct magnetic particles have been found in physiologically and phylogenetically diverse bacteria. Due to a lack of molecular genetic tools, the mechanistic diversity of magnetosome formation remains poorly understood. *Desulfovibrio magneticus* RS-1 is an anaerobic sulfate-reducing deltaproteobacterium that forms bullet-shaped magnetite crystals. A recent forward genetic screen identified 10 genes in the conserved magnetosome gene island of *D. magneticus* that are essential for its magnetic phenotype. However, this screen likely missed mutants with defects in crystal size, shape, and arrangement. Reverse genetics to target the remaining putative magnetosome genes using standard genetic methods of suicide vector integration have not been feasible due to the low transconjugation efficiency. Here, we present a reverse genetic method for targeted mutagenesis in *D. magneticus* using a replicative plasmid. To test this method, we generated a mutant resistant to 5-fluorouracil by making a markerless deletion of the *upp* gene that encodes uracil phosphoribosyltransferase. We also used this method for targeted marker exchange mutagenesis by replacing *kupM*, a gene identified in our previous screen as a magnetosome formation factor, with a streptomycin resistance cassette. Overall, our results show that targeted mutagenesis using a replicative plasmid is effective in *D. magneticus* and may also be applied to other genetically recalcitrant bacteria.

IMPORTANCE Magnetotactic bacteria (MTB) are a group of organisms that form intracellular nanometer-scale magnetic crystals though a complex process involving lipid and protein scaffolds. These magnetic crystals and their lipid membranes, termed magnetosomes, are model systems for studying bacterial cell biology and biomineralization and are potential platforms for biotechnological applications. Due to a lack of genetic tools and unculturable representatives, the mechanisms of magnetosome formation in phylogenetically deeply branching MTB remain unknown. These MTB contain elongated bullet-/tooth-shaped magnetite and greigite crystals that likely form in a manner distinct from that of the cubooctahedral-shaped magnetite crystals of the genetically tractable MTB within the *Alphaproteobacteria*. Here, we present a method for genome editing in *Desulfovibrio magneticus* RS-1, a cultured representative of the deeply branching MTB of the class *Deltaproteobacteria*. This marks a crucial step in developing *D. magneticus* as a model for studying diverse mechanisms of magnetic particle formation by MTB.

KEYWORDS *Desulfovibrio*, biomineralization, genome editing, iron, magnetosomes, magnetotactic bacteria, organelles

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Magnetotactic bacteria (MTB) are a group of diverse microorganisms that align along magnetic fields via their intracellular chains of magnetic crystals (1, 2). Each magnetic crystal consists of either magnetite (Fe_3O_4) or greigite (Fe_3S_4) and is synthesized within a complex organelle called a magnetosome (3). The first cultured MTB were microaerophilic members of the *Alphaproteobacteria*, which form cubooctahedral-shaped magnetite crystals and have served as model organisms for understanding magnetosome formation (4–7). Early studies on *Magnetospirillum* spp. revealed a lipid-bilayer membrane, with a unique suite of proteins, surrounding each magnetite crystal (8–10). The development of genetic tools in *Magnetospirillum magneticum* AMB-1 and *Magnetospirillum gryphiswaldense* MSR-1 revealed a conserved magnetosome gene island (MAI) that contains the factors necessary and sufficient for the formation of the magnetosome membrane, magnetite biomineralization within the lumen of the magnetosome, and alignment of the magnetosomes in a chain along the length of the cell (3, 11). These molecular advances, along with the magnetic properties of magnetosomes, have made MTB ideal models for the study of compartmentalization and biomineralization in bacteria as well as a target for the development of biomedical and industrial applications.

Improvements in isolation techniques and sequencing have revealed that MTB are ubiquitous in many aquatic environments. On the basis of phylogeny and magnetosome morphology, MTB can be categorized into two subgroups. The first subgroup includes members of the *Alphaproteobacteria* and *Gammaproteobacteria*, such as *Magnetospirillum* spp., that synthesize cubooctahedral, elongated octahedral, or elongated prisms of magnetite (12). The second subgroup comprises MTB from more deep-branching lineages, including members of the *Deltaproteobacteria* class and the *Nitrospirae* and *Omnitrophica* phyla, which synthesize elongated bullet-/tooth-shaped magnetite and/or greigite crystals (13, 14). While all MTB sequenced to date have their putative magnetosome genes arranged in distinct regions of their genomes (3, 15–17), many of the genes essential for magnetosome biogenesis in *Magnetospirillum* spp. are missing from the genomes of deep-branching MTB (14). Likewise, a conserved set of *mad* (magnetosome-associated *Deltaproteobacteria*) genes are only found in deep-branching MTB (14, 18–20). This suggests a genetic diversity underpinning the control of magnetosome morphology and physiology in nonmodel MTB that is distinct from that of the well-characterized *Magnetospirillum* spp.

Desulfovibrio magneticus RS-1, one of the few cultured MTB outside the *Alphaproteobacteria*, is an anaerobic sulfate-reducing member of the *Deltaproteobacteria* that forms irregular bullet-shaped crystals of magnetite (21, 22). As with the *Magnetospirillum* spp., the magnetosome genes of *D. magneticus* are located within a MAI and include homologs to some *mam* genes as well as *mad* genes (14, 18, 23). Recently, we used a forward genetic screen combining random chemical and UV mutagenesis with whole-genome resequencing to identify mutations that resulted in nonmagnetic phenotypes. These included many mutants that had the entire MAI deleted (ΔMAI) as well as mutants with point mutations, frameshift mutations, and transposon insertions in 10 *mam* and *mad* genes of the *D. magneticus* MAI that resulted in nonmagnetic phenotypes (20). However, this screen relied on a strict selection scheme for nonmagnetic mutants. As such, we likely missed magnetosome genes that are important for regulating the shape, size, and arrangement of magnetosomes. To elucidate the degree of conservation between *mam* genes and determine the function of the proteins encoded by *mad* genes in *D. magneticus*, a reverse genetic method for targeted mutagenesis is necessary.

D. magneticus and other *Desulfovibrio* spp. have gained much attention for their importance in the global cycling of numerous elements, in biocorrosion, and in the bioremediation of toxic metal ions (24, 25). The development of genetic tools, such as expression vectors, transposons, and targeted genome-editing systems, has enabled a more detailed examination of the important activities of a few *Desulfovibrio* spp. (26, 27). Targeted mutagenesis using a one-step double recombination method was first achieved in *Desulfovibrio fructosivorans* and, more recently, in *Desulfovibrio gigas* and *Desulfovibrio desulfuricans* ND132 (28–30). With this method, plasmids that are electro-

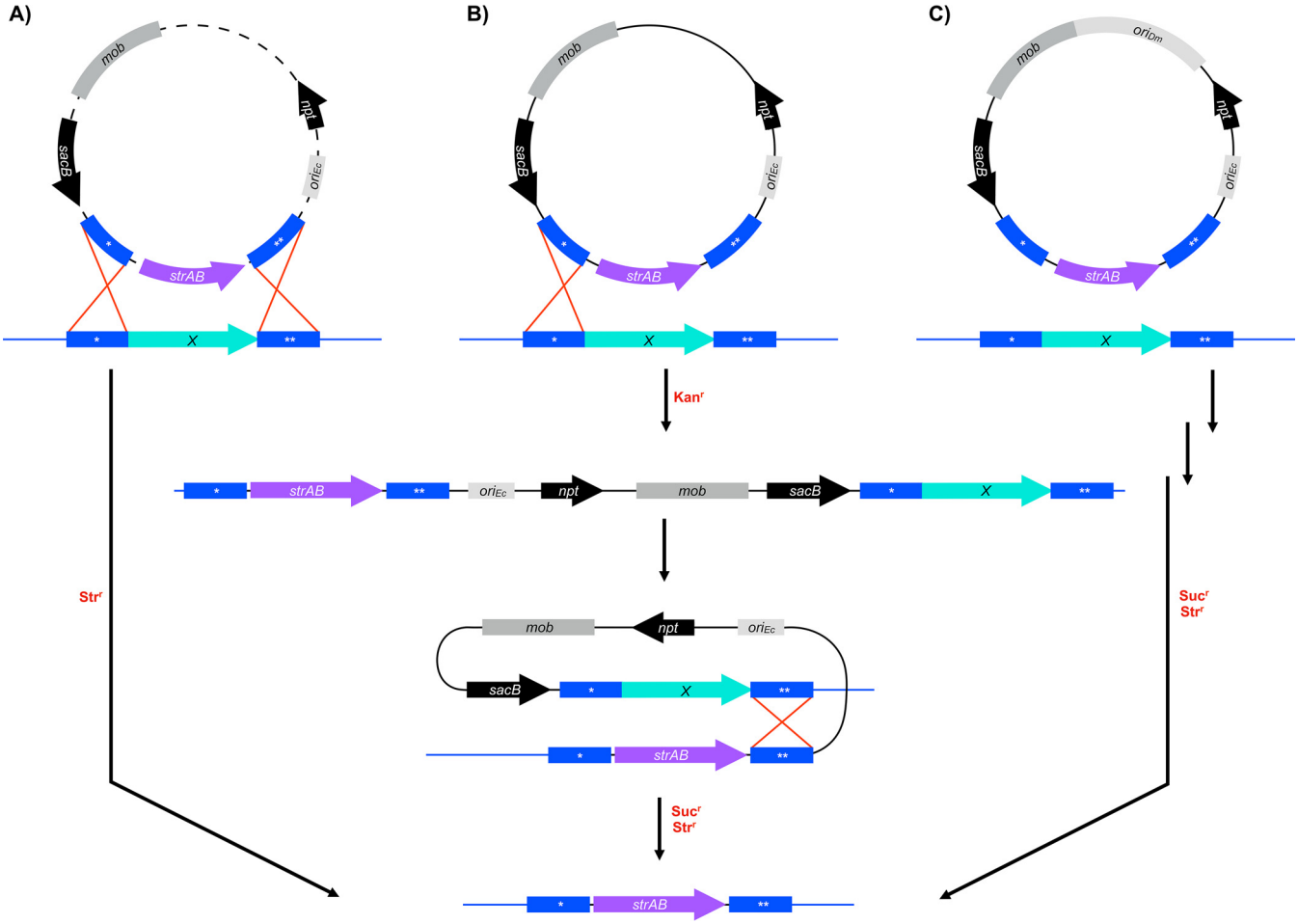

FIG 1 Schematic of deletion methods used in *Desulfovibrio* spp. Plasmids (black lines) were designed to replace a target gene (*X*, aqua arrows) in the chromosome (blue lines) with a streptomycin resistance cassette (*strAB*, purple arrows). Regions upstream (*) and downstream (**) of the target gene (blue boxes) on the chromosome undergo recombination (red lines) with homologous regions that are cloned into the deletion plasmid. Key steps, such as recombination events (red crosses), are indicated in the boxes, and the selection steps are labeled in red. (A) Double recombination can occur in one step after plasmids are linearized (dashed lines) by endogenous restriction enzymes. Mutants are selected using the marker (e.g., *strAB*) that was exchanged with the target gene. (B) Two-step double recombination is possible when suicide vectors integrate into the chromosome in the first homologous recombination event and then recombine out after the second homologous recombination event. The first step and second step are selected for with antibiotic resistance markers (e.g., *npt*) and counterselectable markers (e.g., *sacB*), respectively. (C) A replicative deletion plasmid designed to target genes for deletion may undergo double recombination in one or two steps as shown in panels A and B, respectively. After passaging the cells without antibiotic, the mutants are selected with an antibiotic resistance cassette (e.g., *strAB*) and a counterselectable marker (e.g., *sacB*). *mob*, mobilization genes (*mobA′*, *mobB*, *mobC*); *npt*, kanamycin-resistance gene; ori_{Dm}, origin of replication for *D. magneticus*; ori_{Ec}, origin of replication for *E. coli*.

porated into the cell are thought to be rapidly linearized by endogenous restriction modification systems (30–32). The linearized plasmid DNA, carrying a selectable marker flanked by upstream and downstream regions of homology to a target gene, can then undergo double recombination into the chromosome in one step (Fig. 1A). This efficient one-step method, which is dependent on electroporation of the plasmid (28–30), is unlikely to be applicable for *D. magneticus*, because plasmid uptake has only been demonstrated using conjugal transfer (20). The second targeted mutagenesis method, used in *Desulfovibrio vulgaris* Hildenborough, is a two-step double recombination that makes use of a nonreplicative, or suicide, vector (31, 32). In the first step of this method, a suicide vector, with sequences upstream and downstream of the target gene, integrates into the genome upon the first homologous recombination event (Fig. 1B). Next, a second recombination event occurs whereby the vector is excised from the genome, and cells with the desired genotype are selected with an antibiotic marker and/or a counterselection marker (31, 32) (Fig. 1B). For many bacteria, including *D. magneticus*, plasmid uptake and integration occur at frequencies that are too low for genetic manipulation via suicide vectors (20).

Here, we describe the method we developed for targeted gene deletion using a replicative plasmid, thereby bypassing the need for suicide vector integration (Fig. 1C). We generated a mutant resistant to 5-fluorouracil by making a markerless deletion of the *upp* gene, which encodes an enzyme in the pyrimidine salvage pathway that is nonessential under standard laboratory conditions. Additionally, we deleted *kupM*, a gene encoding a potassium transporter that acts as a magnetosome formation factor (20), via marker exchange with a streptomycin resistance cassette. The deletion of both *upp* and *kupM* conferred the expected phenotypes, which were subsequently complemented in *trans*. Overall, our results show that targeted mutagenesis using a replicative plasmid is possible in *D. magneticus*. It may also be suitable for other bacteria for which replicative plasmid uptake is possible but at a rate too low for suicide vector integration.

RESULTS

Design of a replicative deletion plasmid using *sacB* counterselection. Targeted genetic manipulation in most bacteria requires a method to efficiently deliver foreign DNA destined for integration into the chromosome. One commonly used method involves suicide vector uptake and integration prior to the first selection step (Fig. 1B). In *D. magneticus*, plasmid transfer has only been achieved via conjugation at low efficiencies, making the uptake and subsequent integration of suicide vectors into its chromosome an unlikely event (20). As such, we attempted to bypass the use of suicide vectors and use a stable replicative plasmid designed to delete specific genes via homologous recombination (Fig. 1C). Two features of this method enable the isolation of desired mutants: (i) a selectable marker is used to identify double recombination events at the targeted site and (ii) a counterselectable marker distinguishes the desired mutant cells, which have lost all remaining copies of the plasmid.

sacB is a common counterselection marker that is effective in many bacteria. The *sacB* gene from *Bacillus subtilis* encodes levansucrase, which converts sucrose to levans that are lethal to many Gram-negative bacteria, including *D. vulgaris* Hildenborough (31, 33, 34). To test its functionality in *D. magneticus*, we inserted *sacB* under the expression of the *mamA* promoter of *D. magneticus* (described in reference 20) in a plasmid that replicates in both *Escherichia coli* and *D. magneticus* (Fig. 2A). This plasmid (pAK914) and a control plasmid were then conjugated into *D. magneticus*. We found no growth inhibition for *D. magneticus* cells with the control plasmid in the presence of sucrose and kanamycin. In contrast, cells expressing *sacB* were unable to grow with kanamycin and sucrose concentrations of 1% (wt/vol) or higher (data not shown). To test if the plasmids could be cured, *D. magneticus* with pAK914 was passaged two times in liquid medium containing no antibiotic and plated on 1% sucrose. Individual sucrose-resistant (Suc^r) colonies were inoculated and screened for kanamycin sensitivity (Kan^s). All isolated colonies ($n = 16$) were Kan^s, suggesting that the cells had lost the plasmid. These experiments demonstrate that *sacB* is a suitable counterselection marker in *D. magneticus*.

Construction of a Δ*upp* strain by markerless deletion. To test our replicative deletion method, we chose to target the *upp* gene, the mutation of which has a selectable phenotype. The *upp* gene encodes uracil phosphoribosyltransferase (UPRTase), a key enzyme in the pyrimidine salvage pathway that catalyzes the reaction of uracil with 5-phosphoribosyl-α-1-pyrophosphate (PRPP) to UMP and PP_i (35) (Fig. 3A). When given the pyrimidine analog 5-fluorouracil (5-FU), UPRTase catalyzes the production of 5-fluoroxyuridine monophosphate (5-FUMP). 5-FUMP is further metabolized and incorporated into DNA, RNA, and sugar nucleotides resulting in eventual cell death (Fig. 3A) (36, 37). Previous studies have shown that Δ*upp* mutants of *D. vulgaris* Hildenborough are resistant to 5-FU, while wild-type (WT) cells are effectively killed by the pyrimidine analog (32, 38). The *D. magneticus* genome has a homolog (*DMR_08390*) to the *D. vulgaris* Hildenborough *upp* gene that is likely functional, as detected by the sensitivity of *D. magneticus* to 5-FU (Fig. 3B and 4A). To show that the *upp* gene product

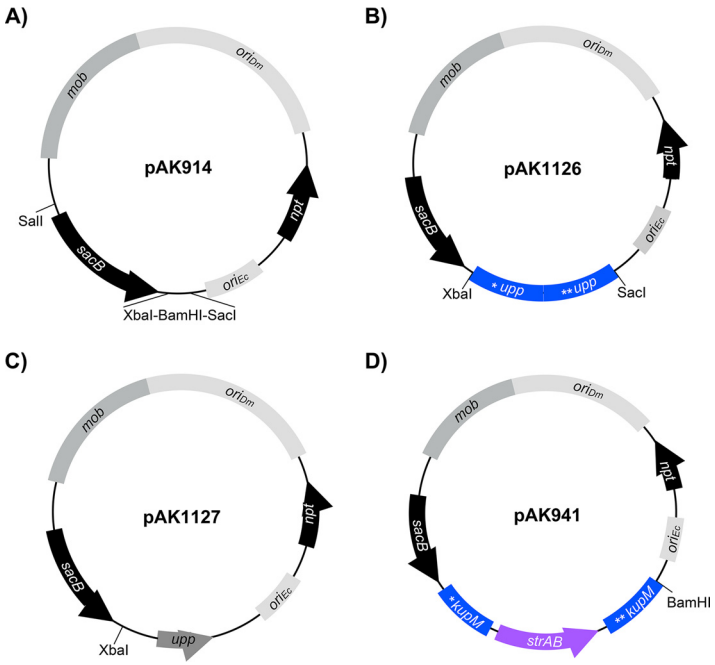

FIG 2 Plasmids constructed for the present study. (A) Expression plasmid pAK914 expresses *sacB* from the *mamA* promoter and is the parent vector for the deletion plasmids and *upp* expression plasmid described below. (B) Replicative deletion plasmid to target *upp* for markerless deletion. The *upp* deletion cassette was cloned into XbaI-SacI of pAK914. (C) Expression plasmid used for *upp* complementation. The *upp* gene and its promoter were cloned into BamHI-SacI of pAK914. (D) Replicative deletion plasmid to target *kupM* for marker exchange mutagenesis with *strAB*. The *kupM*::*strAB* deletion cassette was cloned into XbaI of pAK914. Labeling and colors correspond to those in Fig. 1.

confers 5-FU sensitivity and to validate our replicative deletion system, we chose to target the *D. magneticus upp* gene for markerless deletion.

To construct a *upp* deletion vector, a markerless cassette containing the regions upstream and downstream of the *upp* gene were inserted into plasmid pAK914 (Fig. 2B). The resulting plasmid (pAK1126) was transferred to WT *D. magneticus* by conjugation and single kanamycin-resistant (Kan^r) colonies were isolated and passaged in growth medium containing no antibiotic. Since *D. magneticus* has interesting features independent of its magnetosomes, the same deletion procedure was also carried out in a nonmagnetic strain (ΔMAI) isolated in our previous genetic studies (20). After the third passage, *upp* mutants that had lost the vector backbone were selected for with 5-FU and sucrose. Compared with those obtained using a control plasmid (pAK914), >20-fold more 5-FU-resistant (5-FU^r) mutants were generated using pAK1126 at a frequency of approximately 10^{-6}. PCR of the region flanking the *upp* gene confirmed that the 5-FU^r colonies harboring pAK1126 resulted from a markerless deletion of *upp* (Δ*upp*), while 5-FU^r colonies harboring pAK914 were likely the result of point mutations (Fig. 3B and D). Similar to the results obtained for *D. vulgaris* Hildenborough (32), the Δ*upp* mutant of *D. magneticus* grew in the presence of 5-FU (Fig. 4B and Table 1). Complementation of the *upp* gene in *trans* restored UPRTase function, and the cells no longer grew with 5-FU (Fig. 2C and 4C and Table 1). These experiments demonstrate that a replicative plasmid can be used to directly edit the *D. magneticus* genome.

Construction of a Δ*kup* strain by marker exchange mutagenesis. Because many genetic mutations do not confer a selectable phenotype, we sought to develop our replicative deletion plasmid for marker exchange mutagenesis. To test this system, we chose to replace a gene with a known phenotype, *kupM* (*DMR_40800*), with a streptomycin-resistance gene cassette (*strAB*). *kupM* is located in the *D. magneticus* MAI and encodes a functional potassium transporter (20). Mutant alleles in *kupM*, including missense, nonsense, and frameshift mutations, were previously identified in our screen

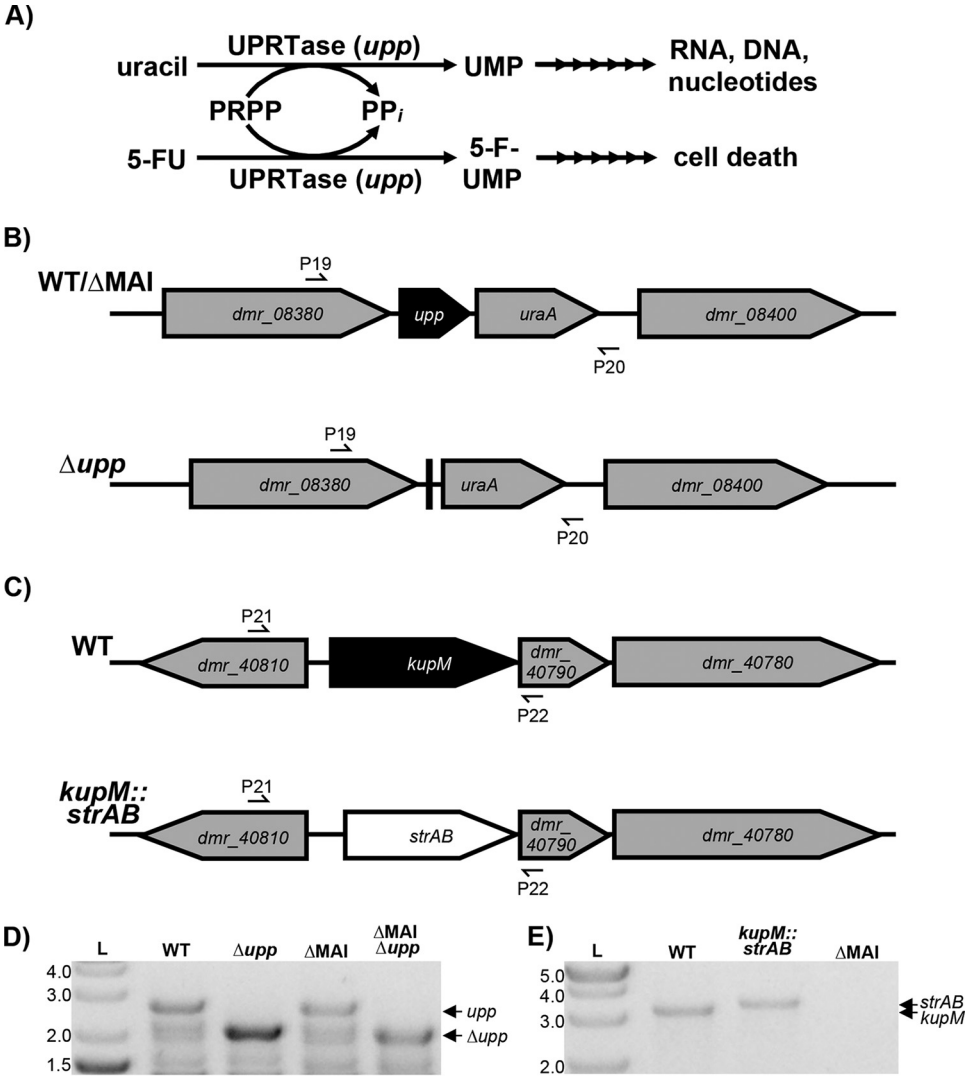

FIG 3 (A) The *upp* gene encodes UPRTase, which is a key enzyme in the uracil salvage pathway. The product of the UPRTase reaction, UMP, is processed by downstream enzymes in pathways for RNA, DNA, and sugar nucleotide synthesis. 5-FU causes cell death by incorporating into this pathway via UPRTase. (B) Schematic of genomic regions of *upp* in the WT or the ΔMAI mutant (top) and the Δ*upp* mutant (bottom). (C) Genomic region of *kup* in WT (top) and *kup*::*strAB* (bottom) strains. Primers used to screen for the correct genotype are indicated with half arrows. (D) Δ*upp* mutants in WT and ΔMAI backgrounds were confirmed by PCR using primers P19/P20 and agarose gel electrophoresis. WT and ΔMAI strains show a band corresponding to the *upp* gene (2,691 bp), while the Δ*upp* mutants have a smaller band corresponding to a markerless deletion of the *upp* gene (2,079 bp). The lower bands are likely nonspecific PCR products. (E) *kupM*::*strAB* genotype confirmation by PCR and agarose gel electrophoresis using primers P21/P22 (WT, 3,069 bp; *kupM*::*strAB*, 3,263 bp; ΔMAI, not applicable [NA]).

for nonmagnetic mutants (20). These *kupM* mutations resulted in cells that rarely contained electron-dense particles and were unable to turn in a magnetic field, as measured by the coefficient of magnetism (C_{mag}) (20).

To mutate *kupM*, we inserted a marker exchange cassette, with regions upstream and downstream of *kupM* flanking *strAB*, into pAK914 (Fig. 2D) to create the deletion plasmid pAK941. Following conjugation, single colonies of *D. magneticus* harboring pAK941 were isolated by kanamycin selection. After three passages in growth medium without selection, potential mutants were isolated at a frequency of approximately 10^{-6} on plates containing streptomycin and sucrose. Single colonies that were streptomycin resistant (Str^r) and Suc^r were inoculated in liquid medium and screened for Kan^s. Of the isolates screened ($n = 48$), 20% were Kan^s and 4% had the correct genotype (Δ*kupM*::*strAB*) as confirmed by PCR and sequencing (Fig. 3C and E).

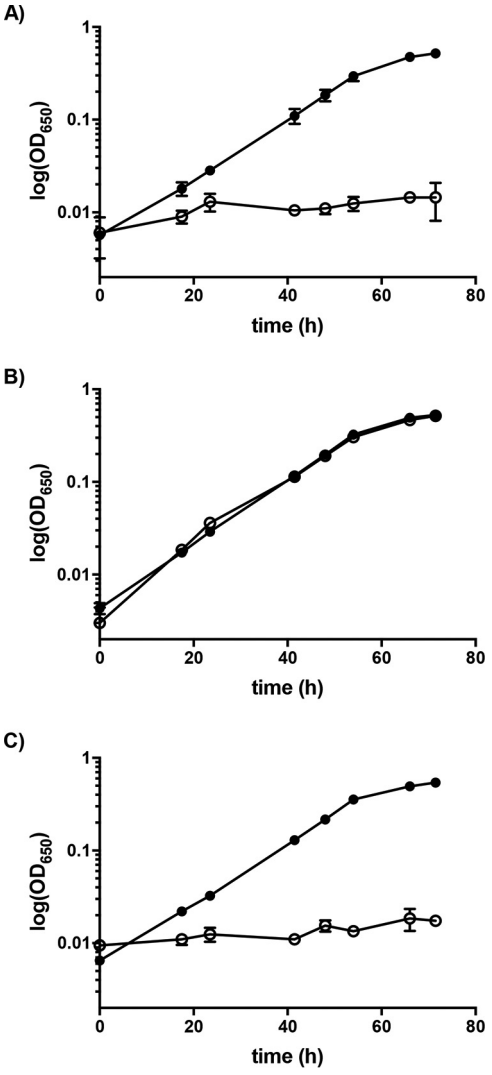

FIG 4 *upp* mutant and complementation phenotype. Growth of the parent strain (ΔMAI) (A), *upp* deletion (ΔMAI Δ*upp*) (B), and complementation of the *upp* deletion (ΔMAI Δ*upp*/*upp*$^+$) (C) when grown with 1.25 μg/ml 5-FU (○) or without 5-FU (●). Data presented are averages from 2 to 3 independent cultures; error bars indicate the standard deviations.

Similar to the phenotypes previously observed in *kupM* mutants (20), Δ*kupM*::*strAB* cells were severely defective in magnetosome synthesis and turning in a magnetic field (Fig. 5). Although a slight C_{mag} was measured, few cells contained electron-dense particles or magnetosomes. Importantly, the WT phenotype was rescued by expressing *kupM* from a plasmid in the Δ*kupM*::*strAB* mutant (Fig. 5). These results confirm that the replicative deletion plasmid method described here can be used successfully for marker exchange mutagenesis.

TABLE 1 Growth rates and generation times of the parent strain (ΔMAI), Δ*upp* mutant, and *upp* complementation in *trans* with and without treatment with 5-FU

Strain	Growth rate (h^{-1})		Generation time (h)	
	Without 5-FU	With 5-FU	Without 5-FU	With 5-FU
ΔMAI	0.077 ± 0.0017	NA[a]	9.1 ± 0.2	NA
ΔMAI Δ*upp*	0.079 ± 0.0017	0.070 ± 0.0040	8.8 ± 0.2	10.0 ± 0.6
ΔMAI Δ*upp*/*upp*$^+$	0.076 ± 0.0041	NA	9.1 ± 0.5	NA

[a]NA, not applicable.

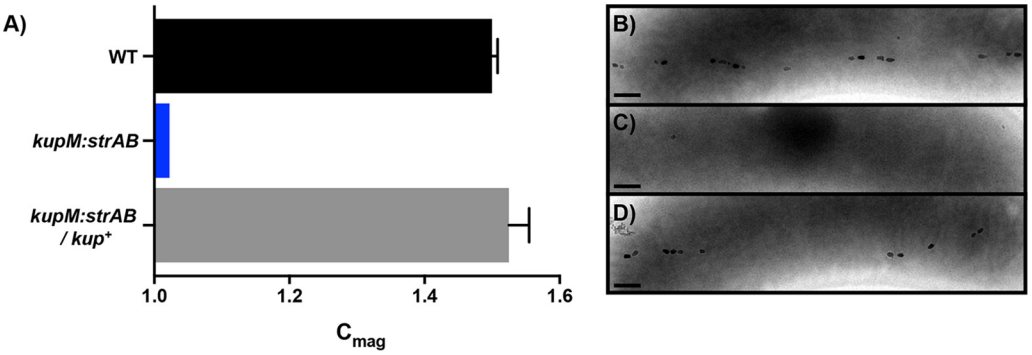

FIG 5 *kupM* mutant and complementation phenotype. C_{mag} values (A) and electron micrographs of WT (B), *kupM*::*strAB* (C), and Δ*kupM*::*strAB*/*kup*$^+$ (D) strains. Scale bars, 200 nm. Data presented are averages from 4 independent cultures; error bars indicate the standard deviations.

DISCUSSION

In this study, we expand the genetic toolbox for *D. magneticus* to include a replicative plasmid method for targeted mutagenesis (Fig. 1C). We show the utility of this method for markerless deletion of genes with a selectable phenotype and for marker exchange mutagenesis. Some of the earliest examples of targeted mutagenesis in Gram-negative bacteria used replicative plasmids, similar to the method described here (34, 39). These studies, which predated the application of suicide vectors, relied on plasmid instability by introducing a second plasmid of the same incompatibility group or by limiting nutrients in the growth medium (34, 39).

Because the *D. magneticus* genetic toolbox has a limited number of plasmids, antibiotic markers, and narrow growth constraints, we used a replicative plasmid and established *sacB* as a counterselection marker to generate and isolate mutants. While *sacB* counterselection was ultimately successful, a large number of false positives were also isolated at the sucrose selection step. Mutations in *sacB* have been found to occur at a high frequency in many bacteria (31, 40–43). Indeed, we found that deletions and mutations in P_{mamA}-*sacB* are abundant in the false-positive Suc^r Str^r isolates (data not shown). Alternative counterselection markers, including *upp*, have been shown to select for fewer false positives (32, 43–45). Since *D. magneticus* is sensitive to 5-FU only when the *upp* gene is present (Fig. 4), the *upp* mutants generated in this study may be used as the parent strains for future targeted mutagenesis with *upp*, rather than *sacB*, serving as a counterselectable marker. Additionally, the combined use of *upp* and *sacB* for counterselection might reduce the false-positive background that results from the accumulation of mutations in these markers.

The replicative deletion plasmid described here was designed to replace a target gene with an antibiotic resistance marker. As such, the construction of strains with multiple directed mutations will be complicated by the need for additional antibiotic-resistance markers, which are limited in *D. magneticus*. These limitations may be overcome by removing the chromosomal antibiotic marker in subsequent steps (34, 46, 47). Ultimately, improvements in conjugation efficiency or methods for electroporation with high transformation efficiency are desired. Similar to the ongoing development of genetics in *D. vulgaris* Hildenborough, the establishment of a suicide vector delivery system in *D. magneticus* will enable more high-throughput targeted mutagenesis and even the construction of markerless deletion mutants (26, 32).

Overall, we demonstrated the utility of a replicative deletion plasmid to generate targeted mutants of *D. magneticus*. This method marks a crucial step in developing *D. magneticus* as a model for the study of anaerobic sulfate reduction and diverse mechanisms of magnetic particle formation by MTB. Both MTB and sulfate-reducing bacteria have been singled out for their role in the global cycling of numerous elements and for potential applications, such as bioremediation (24, 25, 48, 49). *D. magneticus*, in particular, may be useful in the bioremediation of heavy metals and in the global

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
DH5α λpir	Cloning strain	Lab strain
WM3064	Conjugation strain; DAP auxotroph used for plasmid transfer	Lab strain
D. magneticus		
AK80	Nonmotile mutant of *D. magneticus* strain RS-1, referred to as wild type	51
AK201	ΔMAI	20
AK267	ΔMAI Δ*upp*	This study
AK268	Δ*upp*	This study
AK270	Δ*kupM*::*strAB*	This study
Plasmids		
pBMK7	Conjugative vector with pBG1 and pMB1 replicons; Kan^r	53
pBMC7	Conjugative vector with pBG1 and pMB1 replicons; Cm^r	53
pBMS6	Cloning vector; source of *strAB*; Str^r	53
pLR6	pBMK7 with P_{mamA} in HindIII-SalI; source of P_{npt}; Kan^r	20
pLR41	pLR6 with P_{mamA}-*kupM* in SalI; Kan^r	20
pAK0	Cloning vector, source of *sacB*; Kan^r	10
pAK914	pLR6 with *sacB* in SalI-XbaI; Kan^r	This study
pAK920	pBMC7 with P_{npt}-*strAB* inserted into SacI site; Cm^r Str^r	This study
pAK941	pAK914 with cassette of 1,064 bp upstream and 1,057 bp downstream of *kupM* flanking P_{npt}-*strAB* in XbaI; Kan^r Str^r	This study
pAK1126	pAK914 with cassette of 991 bp upstream and 1,012 bp downstream of *upp* in XbaI-SacI; Kan^r	This study
pAK1127	pAK914 with P_{upp}-*upp* in BamHI-SacI; Kan^r	This study

cycling of iron, since it can form both magnetosomes and other iron-containing organelles (50, 51). Through genetic manipulation of *D. magneticus*, pathways of elemental cycling and heavy-metal turnover may now be explored. Additionally, genetic manipulation of *D. magneticus* will further our understanding of magnetosome formation and provide answers to many longstanding questions for the deeply branching MTB. Which proteins regulate and control magnetosome formation? To what extent are lipid membranes involved in forming these crystals? How is the elongated and irregular crystal shape achieved? Finally, in addition to *D. magneticus*, the method described here may extend to other bacteria that are not amenable to targeted mutagenesis with suicide vectors but are able to accommodate replicative plasmids.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 2. All *E. coli* strains were cultured aerobically with continuous shaking at 250 rpm at 37°C in lysogeny broth (LB). *D. magneticus* strains were grown anaerobically at 30°C in sealed Balch tubes with a N_2 headspace containing RS-1 growth medium (RGM) that was degassed with N_2, unless otherwise stated (51). Sodium pyruvate (10 mM) was used as an electron donor with fumaric acid disodium (10 mM) as the terminal electron acceptor. RGM was buffered with HEPES, and the pH was adjusted to 6.7 with NaOH (20). Before inoculating with cells, RGM was supplemented with 0.8% (vol/vol) Wolfe's vitamins, 100 μM ferric malate, and 285 μM cysteine-HCl (51). Solid agar plates were prepared by adding 1.5% agar (wt/vol) to LB and 1% agar (wt/vol) to RGM. Vitamins (0.8% [vol/vol]), ferric malate (20 μM), and cysteine (285 μM), as well as antibiotics and selective agents, were added to the molten RGM agar as needed. For *D. magneticus*, all plating steps were carried out aerobically, and the bacteria were transferred to an anaerobic jar and incubated at 30°C for 10 to 14 days, as described previously (20). The antibiotics and selective agents used are as follows: kanamycin (50 μg/ml for *E. coli* strains, 125 μg/ml for *D. magneticus* strains), streptomycin (50 μg/ml for *E. coli* and *D. magneticus* strains), diaminopimelic acid (300 μM for *E. coli* WM3064), 5-FU (2.5 μg/ml for *D. magneticus* strains), and sucrose (1% for *D. magneticus* strains).

Plasmids and cloning. All plasmids used in this work are listed in Table 2. All cloning was performed in *E. coli* DH5α λpir using the Gibson method (52) or restriction enzyme ligation. For PCR amplification, KOD (EMD Millipore, Germany) and GoTaq (Promega, USA) DNA polymerases were used with the primers listed in Table 3. All upstream and downstream homology regions were amplified from *D. magneticus* genomic DNA. *strAB* and P_{npt} were amplified from pBMS6 and pLR6, respectively, and subcloned into pBMC7 to make pAK920, which served as the template for amplifying P_{npt}-*strAB* for the deletion vectors. *sacB* was amplified from pAK0 and inserted into pLR6 digested with SalI and XbaI to create pAK914. To construct a plasmid for the targeted deletion of *upp* (*DMR_08390*), 991 bp upstream and 1,012 bp downstream of *upp* were amplified and inserted into pAK914 digested with XbaI and SacI using a 3-piece

TABLE 3 Primers used in this study

Name	Sequence from 5′ end	Description[a]
P1	AAGCCAAGAAAAACGTCGCCAACGTCGACATGAACATCAAAAAGTTTGCA	F *sacB* for pAK914
P2	GCTCGGTACCCGGGGATCCTCTAGAGGCCAATAGGATATCGGCATTT	R *sacB* for pAK914
P3	CGACTCTAGAGGATCCCCGGGTACCGTAGCTTCACGCTGCCGCAAG	F P_{npt} for pAK920
P4	CCCGAATGTGCATGCGAAACGATCCTCATCCTGTC	R P_{npt} for pAK920
P5	AGGATCGTTTCGCATGCACATTCGGGATATTTCTCTA	F *strAB* for pAK920
P6	TAATACGACTCACTATAGGGAATTCGCCCAGGGGATAGGAGAAGTC	R *strAB* for pAK920
P7	AAATGCCGATATCCTATTGGCCTCTAGAGAGATCGCGAAGCAGAGC	F *kupM* upstream for pAK941
P8	TGCGGCAGCGTGAAGCTACGGTACCGCCGTAATGCGTCAGAAAGT	R *kupM* upstream for pAK941
P9	CTTCTCCTATCCCCTGGGCGAATTCAGCCGGGTCATGGAAGTC	F *kupM* downstream for pAK941
P10	CGAGCTCGGTACCCGGGGATCCTCTAGAGGCCAGGGAATGGAGTTT	R *kupM* downstream for pAK941
P11	GGTACCGTAGCTTCACGCTGCCGCA	F P_{npt}-*strAB* for pAK941
P12	GAATTCGCCCAGGGGATAGGAGAAGTCGCT	R P_{npt}-*strAB* for pAK941
P13	GCCGATATCCTATTGGCCTCTAGAGCCTCCCAGATCGACCAGTC	F *upp* upstream for pAK1126
P14	CTATTTGGTGCCGGATCCCATGGACGCGCTCCTGGG	R *upp* upstream for pAK1126
P15	AGCGCGTCCATGGGATCCGGCACCAAATAGGGGG	F *upp* downstream for pAK1126
P16	CGACTCACTATAGGGAATTCGAGCTCGCCAGGCAGACGGCGGTG	R *upp* downstream for pAK1126
P17	GCCGATATCCTATTGGCCTCTAGAGAAGCTCGCCGAAAAGACC	F P_{upp}-*upp* for pAK1127
P18	CGACTCACTATAGGGAATTCGATGAAGGCGAACGAGGAAC	R P_{upp}-*upp* for pAK1127
P19	GCCCGCATTGAGGACGTG	To check *upp* deletion
P20	CAGCGCCCCGAGCTTGCC	To check *upp* deletion
P21	CGTCAGCAGGCAAACGG	To check *kupM* deletion
P22	ACCGTTGTCTCCCATGTCTC	To check *kupM* deletion

[a]F, forward; R, reverse.

Gibson assembly. To create the *upp* complementation plasmid, pAK914 was digested with BamHI and SacI, and the *upp* gene, with its promoter, was PCR amplified from *D. magneticus* genomic DNA. To construct pAK941 for marker exchange mutagenesis of *kupM*, a cassette of a 1,064-bp upstream region and 1,057-bp downstream region flanking P_{npt}-*strAB* was assembled using Gibson cloning. The cassette was amplified and inserted into pAK914 digested with XbaI using a two-piece Gibson assembly.

***upp* and *kup* mutant generation and complementation.** Replicative deletion plasmids were transformed into *E. coli* WM3064 by heat shock and transferred to *D. magneticus* by conjugation, as described previously (20). Single colonies of Kan^r *D. magneticus* were isolated and inoculated in RGM containing no antibiotic. Cultures were passaged and, after the third passage, approximately 2×10^8 cells were spread on 1% agar RGM plates containing either 50 μg/ml streptomycin and 1% sucrose or 2.5 μg/ml 5-FU and 1% sucrose. 5-FU^r Suc^r and Str^r Suc^r colonies harboring plasmids pAK1126 and pAK941, respectively, were recovered at a frequency of approximately 10^{-6}. Single colonies were screened for Kan^s and by PCR using the primers listed in Table 3. Successful *upp* and *kup* mutants were confirmed by Sanger sequencing. The expression plasmids for the complementation of Δ*kup*::*strAB* and Δ*upp*, as well as empty vectors for controls, were transferred to *D. magneticus* strains as described above. Transconjugants were inoculated in RGM containing kanamycin to maintain the plasmids.

Mutant phenotype and complementation analyses. The growth and coefficient of magnetism (C_{mag}) of *D. magneticus* strains were measured in a Spec20 spectrophotometer at an optical density of 650 nm (OD_{650}), as described previously (10, 51). For *upp* mutant and complementation analyses, RGM was supplemented with 5-FU (1.25 μg/ml in 0.01% dimethyl sulfoxide [DMSO]) or DMSO (0.01%), and the growth was measured for WT and Δ*upp* strains with an empty vector (pAK914) and for the Δ*upp* strain with the complementation plasmid pAK1127. For *kup* mutant and complementation analyses, the C_{mag} was measured by placing a large bar magnet parallel or perpendicular to the sample to measure the maximum or minimum absorbance, respectively, as the *D. magneticus* strains rotate 90° with the magnetic field. The ratio of maximum to minimum absorbances was calculated as the C_{mag} (10). Whole-cell transmission electron microscopy (TEM) was performed as previously described (51). The C_{mag} calculations and TEM were performed for WT *D. magneticus* with an empty vector (pBMK7) and the Δ*kup*::*strAB* strain with an empty vector (pBMK7) or complementation plasmid (pLR41). For all growth measurements, C_{mag} measurements, and TEM, the cells harboring the plasmids were maintained with 125 μg/ml kanamycin.

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