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Development of a Markerless Deletion Mutagenesis System in Nitrate-Reducing Bacterium *Rhodanobacter denitrificans*

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ABSTRACT *Rhodanobacter* has been found as the dominant genus in aquifers contaminated with high concentrations of nitrate and uranium in Oak Ridge, TN, USA. The *in situ* stimulation of denitrification has been proposed as a potential method to remediate nitrate and uranium contamination. Among the *Rhodanobacter* species, *Rhodanobacter denitrificans* strains have been reported to be capable of denitrification and contain abundant metal resistance genes. However, due to the lack of a mutagenesis system in these strains, our understanding of the mechanisms underlying low-pH resistance and the ability to dominate in the contaminated environment remains limited. Here, we developed an in-frame markerless deletion system in two *R. denitrificans* strains. First, we optimized the growth conditions, tested antibiotic resistance, and determined appropriate transformation parameters in 10 *Rhodanobacter* strains. We then deleted the *upp* gene, which encodes uracil phosphoribosyltransferase, in *R. denitrificans* strains FW104-R3 and FW104-R5. The resulting strains were designated R3_Δ*upp* and R5_Δ*upp* and used as host strains for mutagenesis with 5-fluorouracil (5-FU) resistance as the counterselection marker to generate markerless deletion mutants. To test the developed protocol, the *narG* gene encoding nitrate reductase was knocked out in the R3_Δ*upp* and R5_Δ*upp* host strains. As expected, the *narG* mutants could not grow in anoxic medium with nitrate as the electron acceptor. Overall, these results show that the in-frame markerless deletion system is effective in two *R. denitrificans* strains, which will allow for future functional genomic studies in these strains furthering our understanding of the metabolic and resistance mechanisms present in *Rhodanobacter* species.

IMPORTANCE *Rhodanobacter denitrificans* is capable of denitrification and is also resistant to toxic heavy metals and low pH. Accordingly, the presence of *Rhodanobacter* species at a particular environmental site is considered an indicator of nitrate and uranium contamination. These characteristics suggest its future potential application in bioremediation of nitrate or concurrent nitrate and uranium contamination in groundwater ecosystems. Due to the lack of genetic tools in this organism, the mechanisms of low-pH and heavy metal resistance in *R. denitrificans* strains remain elusive, which impedes its use in bioremediation strategies. Here, we developed a genome editing method in two *R. denitrificans* strains. This work marks a crucial step in developing *Rhodanobacter* as a model for studying the diverse mechanisms of low-pH and heavy metal resistance associated with denitrification.

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Nitrate has been recognized as one of the most prevalent groundwater contaminants, and nitrate contamination in drinking water has become a global environmental issue (1). In addition, as nitrate can abiotically oxidize insoluble uranium(IV) to produce aqueous uranium(VI) (2–4), the coexistence of uranium(VI) and nitrate has been found not only in nuclear legacy wastewater but also in major aquifers in the United States (5, 6). To attenuate nitrate contamination in groundwater, denitrification is generally considered the most promising strategy, which is intimately linked to the presence and activity of denitrifying bacteria (1, 6).

The S-3 disposal ponds located at the U.S. Department of Energy Field Research Center (ORFRC) in Oak Ridge, TN, were highly contaminated by nitrate, uranium, and other heavy metals, due to the deposition of nitric acid-solubilized uranium waste and other mixed metal and organic waste from the Y-12 nuclear processing plant (7, 8). The ponds were later capped and turned into a parking lot. Monitoring of the surrounding groundwater revealed a high concentration of nitrate (up to 0.7 M), uranium (0.7 mM), and other metals including aluminum (20.1 mM), manganese (3.1 mM), and nickel (0.2 mM) (7, 9, 10). To better design future bioremediation strategies in this contaminated area, several studies focusing on characterizing the microbial community composition in numerous wells at the ORFRC were conducted (11–16). The data showed that *Rhodanobacter* species were dominant in the microbial communities in the most contaminated wells where the nitrate concentrations were higher than 5 mM, uranium concentrations were higher than 2.5 μM, and the pH of the groundwater was below 4 (13, 15). Consequently, *Rhodanobacter* has been considered an indicator of contamination at this field site (11, 13, 16).

Rhodanobacter species (class: *Gammaproteobacteria*) are Gram-negative, facultative anaerobic bacteria. Certain strains of *Rhodanobacter* can grow at a pH as low as 3, and genome sequencing revealed the presence of a large number and variety of metal resistance genes (6, 15, 17–19). Although denitrification has not been considered a defining property of *Rhodanobacter*, many species, such as *Rhodanobacter denitrificans*, possess the necessary genes to carry out denitrification based on genome annotations (17). *Rhodanobacter* has gained much attention due to its importance in denitrification and heavy metal resistance under low-pH conditions (12, 15–17), and multiple *R. denitrificans* strains have been recently isolated from the ORFRC site. However, it remains challenging to interrogate the mechanisms of denitrification and heavy metal resistance due to the lack of a genetic editing method for *Rhodanobacter* strains.

Here, we aimed to develop an in-frame deletion mutagenesis system in *R. denitrificans*. We started with a systematic characterization of the optimal growth conditions and antibiotic resistance profiles for 10 *R. denitrificans* strains originally isolated from the ORFRC site. Next, we determined the optimal parameters for successful electroporation. Finally, two strains, FW104-R3 and FW104-R5, with similar genome sequences but different acidic tolerances were selected as representatives for development of an in-frame deletion mutagenesis system. The host strains were generated by knocking out the *upp* gene, which encodes uracil phosphoribosyltransferase, and 5-fluorouracil (5-FU) resistance was used as a counterselection marker for the in-frame deletion mutants. Deletion mutants of *narG*, which encodes a nitrate reductase, were constructed, and the resulting mutants were unable to grow in anoxic medium with nitrate as the electron acceptor, demonstrating the in-frame deletion mutagenesis system is an effective strategy in these strains. The development of a mutagenesis approach marks a crucial step in establishing *R. denitrificans* as a model organism for studying the diverse mechanisms of denitrification for bioremediation of contaminated groundwater and global nitrogen cycling under low-pH conditions.

RESULTS

Optimization of growth conditions. To determine the optimal growth conditions of *Rhodanobacter denitrificans* strains, three types of media including tryptic soy agar

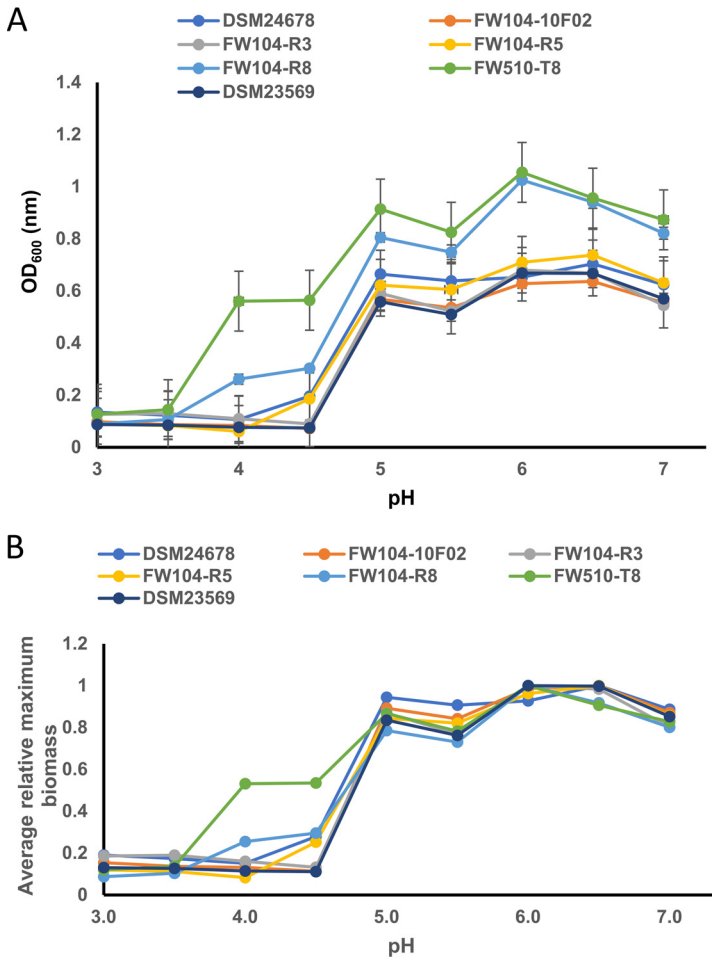

FIG 1 Growth profiles of *Rhodanobacter* strains grown in SGW medium with pH ranging from 3.0 to 7.0. (A) Maximum optical density at 600 nm (OD_{600}). Data are presented as the mean for biological replicates ($n = 4$), and error bars represent standard deviations. (B) Average maximum biomass yield relative to the average maximum OD_{600} under different pH conditions. Since the FW104-T7 and FW104-R12 strains did not grow very well and FW104-R10 could not grow on the R2A/TSA/SGW plates (see Fig. S1 and S2 and Table S1 in the supplemental material), these three strains were excluded from the growth profile determination experiment.

(TSA) medium, Reasoner's 2A agar (R2A) medium, and synthetic groundwater (SGW) medium were tested. These media contain various amounts of nutrients, and growth was tested at a pH range from 6.5 to 7.2. When grown on plates, the optimal pH was 7.2 for R2A, 6.5 for TSA, and 6.5 for SGW for all tested strains (see Table S1 and Fig. S1 in the supplemental material). When grown in liquid media, all strains grew better in R2A and SGW than TSA (Fig. S2), which was different from the growth on plates. SGW medium contains fewer nutrients than R2A and better mimics the environment these *R. denitrificans* strains were isolated from (6). Therefore, SGW was chosen as the medium for further tests. The optimal pH for growth in liquid SGW was 6.0 to 6.5 (Fig. 1 and Fig. S3). The maximum biomass at pH 4.5 decreased by more than 50% compared to the optimal pH, indicating that pH 4.5 is the threshold for growth inhibition (Fig. 1). Therefore, based on the maximum biomass at pH 4.5 (Fig. 1), the strains were categorized into three groups (high-, medium-, and low-acid resistance). Strain FW510-T8 was the only strain in the high-acid resistance group. Its biomass decreased by 50% at pH 4.5 and remained stable at pH 4.0. Three strains including FW104-R5, FW104-R8, and DSM24678 were categorized in the medium-acid tolerance group; their growth was inhibited at pH 4.5, with a biomass decrease of 70% compared to their highest maximum biomass at optimal growth (pH 6.5). Three strains including FW104-R3, FW104-10F02,

TABLE 1 Antibiotic resistance test of the type strain DSM23569[a]

Antibiotic	Result for concn:			
	20 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL
Kanamycin	+	−	−	−
Spectinomycin	+	+	+	+
Erythromycin	+	−	−	−
Chloramphenicol	+	+	−	−
Tetracycline	−	−	−	
Ampicillin	+	+	+	+
Gentamicin[b]	−	−		

[a]The antibiotic resistance test was conducted in R2A medium (pH 7.2). +, growth; −, no growth.
[b]The two tested concentrations were 8 and 15 μg/mL, respectively.

and DSM23569 were in the low-acid tolerance group as all these strains barely grew at pH 4.5. Interestingly, FW104-R5 and FW104-R3 share high genome sequence identity (average nucleotide identity = 99.68%) (19) but showed different acid tolerances, revealing the complexity of pH resistance mechanisms.

Determination of antibiotic resistance profile and transformation parameters. The antibiotic resistance profile and transformation parameters, which are indispensable for constructing a genomic editing system, were determined. We first tested the sensitivity of the type strain DSM23569 to seven different antibiotics. Strain DSM23569 was sensitive to four antibiotics including kanamycin, erythromycin, tetracycline, and gentamicin (Table 1). Consistently, other *Rhodanobacter* strains were also sensitive to kanamycin (50 μg/mL) and gentamicin (15 μg/mL) (Fig. S4). Next, we tested electroporation parameters using two shuttle vectors and one transposon vector to establish a working transformation protocol. The shuttle vector pPROBE-GT harbors a gentamicin resistance gene, and the shuttle vector pBBR1MCS-5 harbors a kanamycin resistance gene. Two voltages, 1,250 V and 1,750 V, were tested. The results demonstrated that higher transformation efficiencies were obtained for most of the strains using the higher voltage (1,750 V) (Table 2). We also tested whether the addition of a type I restriction inhibitor improved the transformation efficiencies since *Rhodanobacter* species have complex restriction-modification (RM) systems, especially an abundance of type I restriction-modification system genes (19). The addition of a type I restriction inhibitor significantly improved the transformation efficiencies (numbers of transformants were 36 $\pm$ 4 and 14 $\pm$ 4 per μg of pPROBE-GT, with or without type I restriction inhibitor, respectively; $P < 0.05$, unpaired two-tailed *t* test) (Fig. S5). Using the optimized electroporation parameters, transformation of a Tn*5*-barcoded transposon system was conducted to test the efficiency of transformation and genome insertions (Fig. S6). The transformation efficiency was ~1,000 clones per μg of Tn*5*-barcoded transposon vector. The transformation efficiencies of both shuttle vector and transposon vector in *R. denitrificans* strains were relatively low compared to those in other model bacteria such as *Escherichia coli*.

TABLE 2 Summary of the transformation efficiencies in different *Rhodanobacter* strains[a]

Strain	Medium	pPROBE_GT		pBBR1MCS-5, 1,750 V
		1,250 V	1,750 V	
DSM24678	SGW, pH 6.5	+	−	++
FW104-R3	SGW, pH 6.5	+	++	++
FW104-R8	SGW, pH 6.5	+	+	+
FW510-T8	SGW, pH 6.5	+	+	−
DSM-23569	R2A, pH 7.2	+	++	+
FW104-R5	R2A, pH 7.2	++	+++	+++
FW104-10F02	R2A, pH 7.2	++	+++	+++
FW510-R12	R2A, pH 7.2	−	+	++

[a]The optimal medium based on growth in liquid medium (Fig. S2) was used for the transformation efficiency test in each strain. Symbols: −, no colony; +, number of colonies between 0 and 25; ++, number of colonies between 25 and 75; +++, number of colonies greater than 75.

Construction of a Δ*upp* host strain. The use of the purine and pyrimidine salvage enzymes, phosphoribosyltransferases (PRTases), as a counterselection strategy is common in many bacterial genetic editing systems (20). Genome analysis of the *R. denitrificans* strains revealed a gene annotated as uracil PRTase, *upp*, indicating that these strains might be sensitive to the toxic pyrimidine analog 5-fluorouracil (5-FU). Two strains including FW104-R5 and FW104-R3 were chosen as target strains for construction of Δ*upp* host strains as they had a high similarity of genome sequence but different acid tolerances. When grown in SGW media containing different concentrations of 5-FU, the growth rate and maximum biomass of FW104-R5 and FW104-R3 were significantly inhibited by $\geq$50 μg/mL of 5-FU ($P < 0.05$, unpaired two-tailed *t* test) (Fig. S7). Therefore, we chose SGW supplemented with 5-FU (100 μg/mL) as the counterselection condition. To generate Δ*upp* parental strains, a suicide vector, pMD-upp, containing ~1,800 bp upstream and ~1,800 bp downstream of the *upp* gene, the gentamicin resistance marker, and the pUC19 ori (Fig. 2A), was constructed and electroporated into strains FW104-R5 and FW104-R3. By selecting gentamicin-resistant clones, transformants with the integration of pMD-upp into the chromosome were obtained (Fig. 2A). These mutants were then grown in SGW without gentamicin for about 24 h to allow the occurrence of the second recombination event. 5-FU^r colonies were then selected as the potential Δ*upp* host strain (Fig. 2A). Sanger sequencing of the PCR-amplified fragments using different sets of primers located inside and outside the homologous arms verified the successful deletion of the open reading frame of *upp* (Fig. 2B and Fig. S8). The resulting mutants were designated R3_Δ*upp* and R5_Δ*upp*. As expected, R3_Δ*upp* and R5_Δ*upp* had a higher growth rate and higher maximum biomass than the wild-type strains when grown in SGW supplemented with different concentrations of 5-FU ($P < 0.05$, unpaired two-tailed *t* test) (Fig. S7). Therefore, R3_Δ*upp* and R5_Δ*upp* were selected as host strains for the generation of markerless deletion mutants.

Construction of a Δ*narG* mutant by markerless deletion. The *narG* gene was chosen as the gene of interest for markerless deletion as it encodes a nitrate reductase, a key enzyme in denitrification. We expected a Δ*narG* strain to be defective in denitrification and unable to grow in anoxic medium with nitrate as the sole electron acceptor. The first step was the construction of a marker exchange (ME) mutant of *narG*. Here, we aimed to delete the *narG* promoter region (200-bp upstream start codon) and the first 600 bases of the *narG* open reading frame to inactivate *narG*. A marker exchange vector, pME-narG, was constructed with parts including an ~1,800-bp homologous region upstream of the potential promoter of the *narG* gene, an ~1,800-bp homologous region which was 600 bp downstream of the *narG* start codon, the *upp* gene amplified from FW104-R3/R5 genomic DNA (gDNA), the kanamycin resistance gene from pMO728, and the gentamicin resistance gene and pUC19 ori from pMD-upp. After transformation of pME-narG into R3_Δ*upp* and R5_Δ*upp* by electroporation, single colonies resistant to kanamycin (Kan^r) were isolated (Fig. 3A). A second round of selection for Gen^r of the Kan^r clones was performed to confirm the integration of the plasmid pME-narG into the chromosome. The Kan^r/Gen^r clones were inoculated into SGW without antibiotics to allow the occurrence of the second recombination event. Either 5-FU^s, Gen^s, and Kan^r colonies harboring replacement of the partial *narG* open reading frame by Kan^r-*upp* cassette (marker) or 5-FU^r, Gen^s, and Kan^s colonies harboring the wild-type *narG* were generated from the second recombination events. The potential marker exchange mutants were selected as Kan^r and verified by PCR amplification of the entire homologous region (Fig. 3B). We selected 10 Kan^r clones, nine were identified as Kan^r Gen^r in the first recombination event, and three clones out of 40 Kan^r clones from the second recombination event were confirmed as ME mutants via PCR amplification. These marker exchange mutants, R3 Δ*upp* Δ*narG*::kan-upp and R5 Δ*upp* Δ*narG*::kan-upp, were used for construction of markerless deletion (MD) of *narG*.

The markerless vector pMD-narG containing the same homologous arms as pME-narG was constructed by Gibson assembly (Fig. 4A). pMD-narG was then electroporated into R3 Δ*upp* Δ*narG*::kan-upp and R5 Δ*upp* Δ*narG*::kan-upp. Similar to the mutant selection

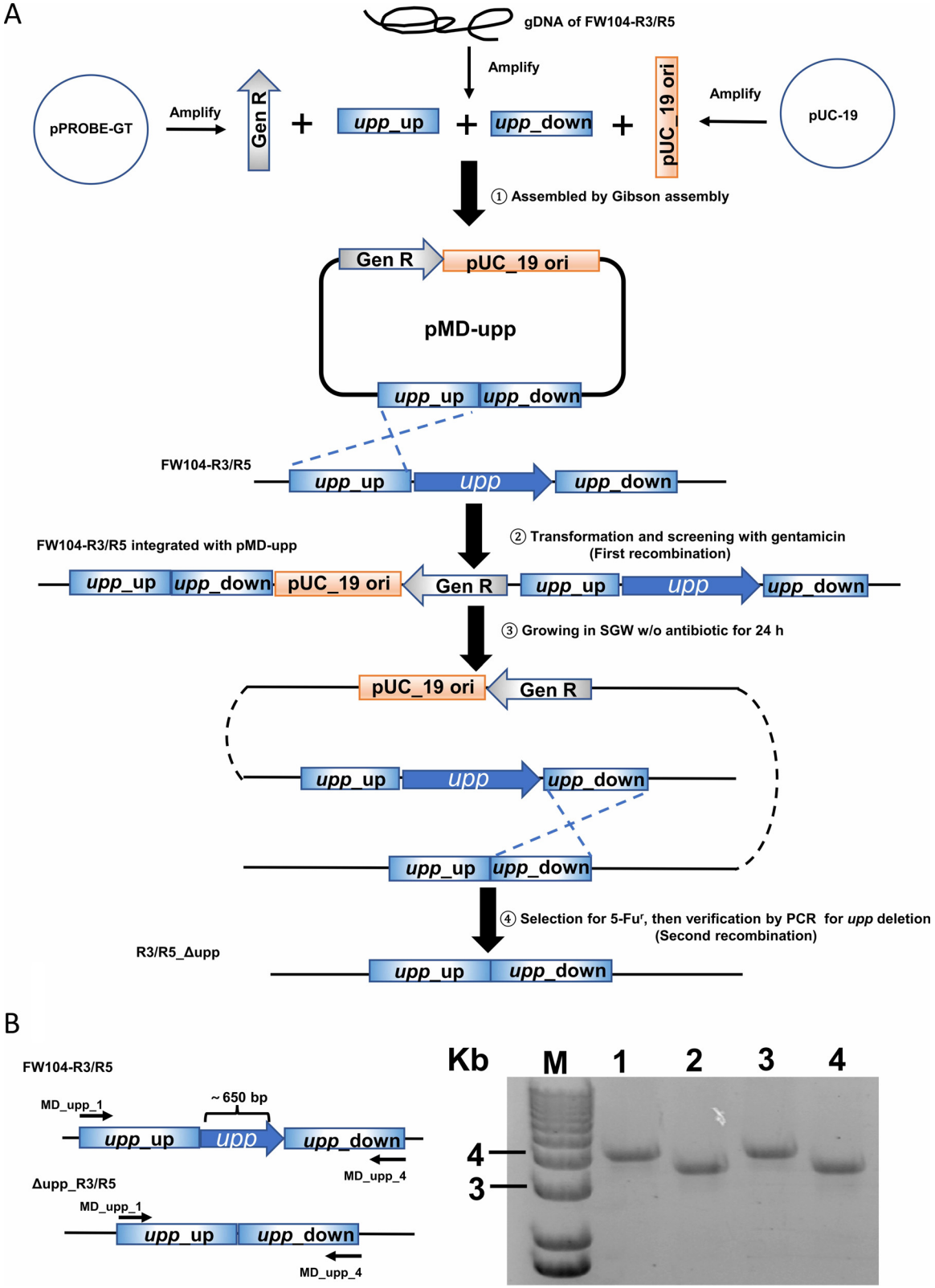

FIG 2 Generation of the Δ*upp* host strains in FW104-R3 and FW104-R5. (A) Schematic of *upp* deletion method. (B) PCR verification of R3/R5_Δ*upp* mutants. Lane 1, FW104-R3; lane 2, R3_Δ*upp*; lane 3, FW104-R5; lane 4, R5_Δ*upp*. M: 1kb plus DNA ladder (Invitrogen).

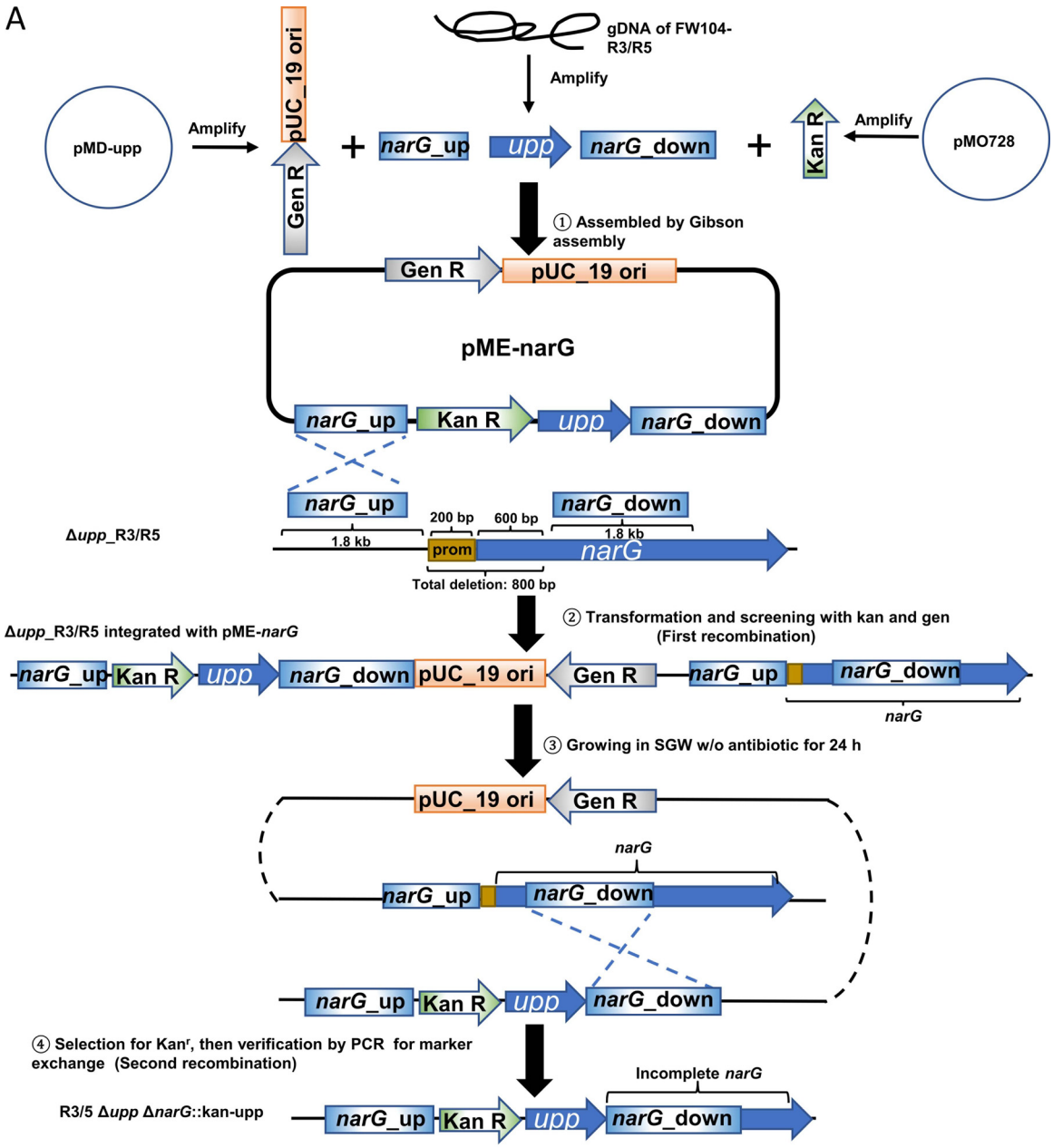

FIG 3 Generation of the Δ*narG* marker exchange strains in R3_Δ*upp* and R5_Δ*upp*. (A) Schematic of marker exchange for *narG*. (B) PCR verification of R3/R5_Δ*upp*_Δ*narG*(ME) mutants. The potential promoter (200 bp) and first 600 bp of *narG* are exchanged with the marker. Lane 1, R3_Δ*upp*; lane 2, R3_Δ*upp*_Δ*narG*(ME); lane 3, R5_Δ*upp*; lane 4, R5_Δ*upp*_Δ*narG*(ME). M: 1kb plus DNA ladder (Invitrogen).

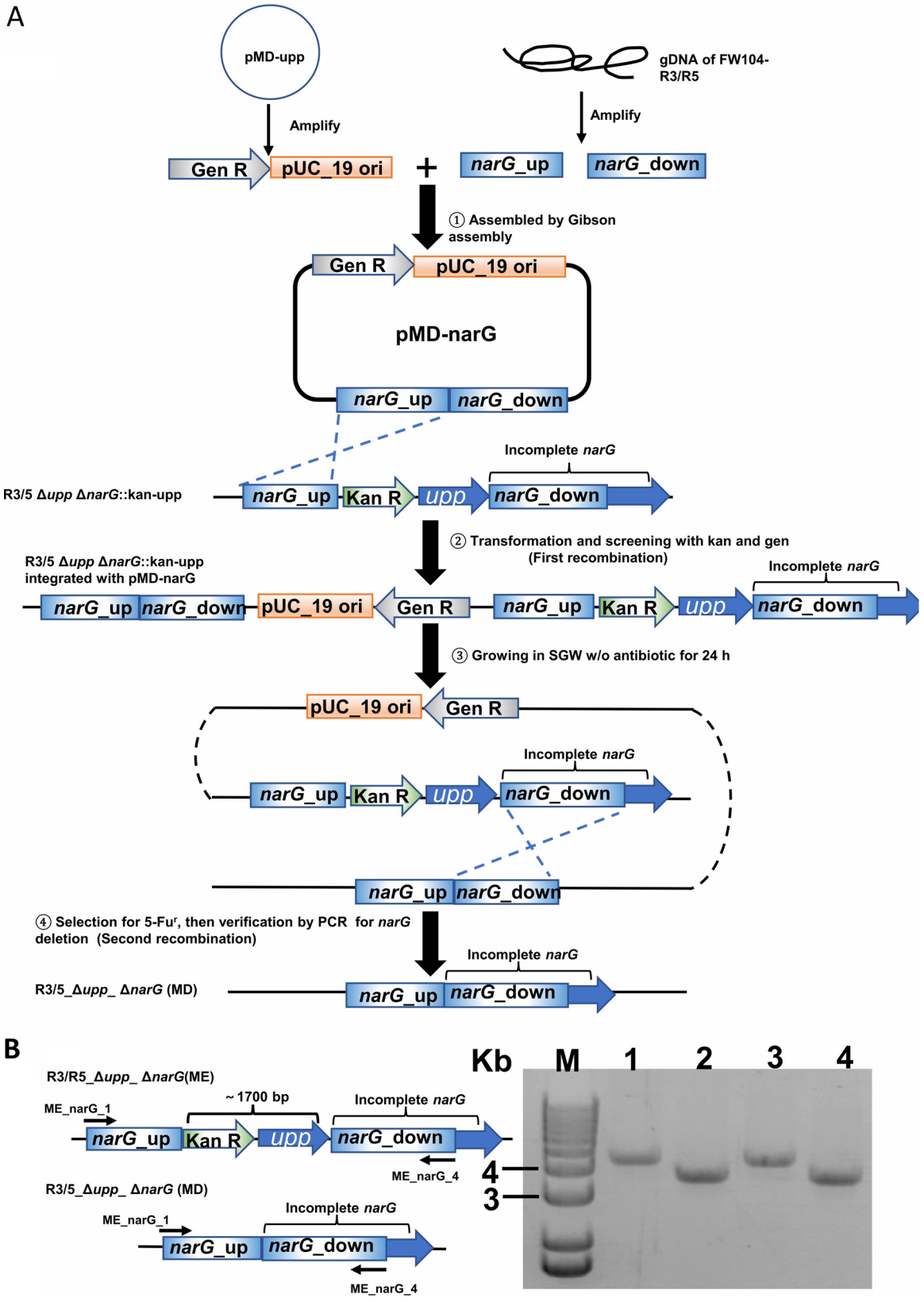

FIG 4 Generation of the Δ*narG* markerless strains in strains R3_Δ*upp*_Δ*narG*(ME) and R5_Δ*upp*_Δ*narG*(ME). (A) Schematic of *narG* markerless deletion method. (B) PCR verification of R3/R5_Δ*upp*_Δ*narG*(MD) mutants. Lane 1, R3_Δ*upp*; lane 2, R3_Δ*upp*_Δ*narG*(MD); lane 3, R5_Δ*upp*; lane 4, R5_Δ*upp*_Δ*narG*(MD). M: 1kb plus DNA ladder (Invitrogen).

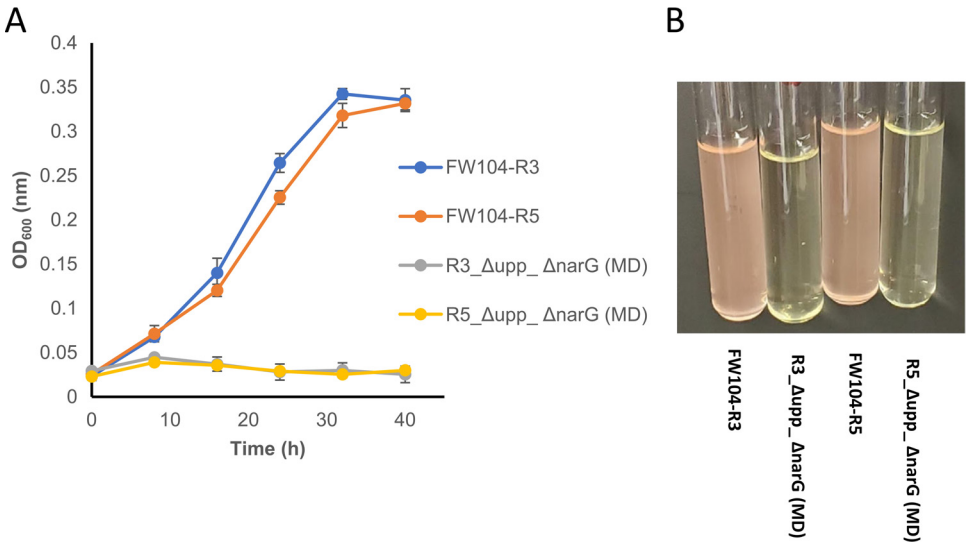

FIG 5 Anaerobic growth analysis of Δ*narG*(MD) mutants with the host strains (R3/R5_Δ*upp*) as controls. Growth curves (A) and pictures of the cultures (B) are shown. Data are presented as the mean from biological replicates (n = 3), and error bars represent standard deviations. The pink color in the medium may be caused by a reaction between resazurin (oxygen indicator) and reactive nitrogen from denitrification.

procedure described above, single colonies resistant to both kanamycin and gentamicin (Kan^r and Gen^r) were selected first, in which the markerless vector was integrated into the chromosomes (Fig. 4A). To allow the occurrence of the second recombination event and the resulting loss of marker (kana^r-*upp* cassette) from the chromosome, the individual Kan^r Gen^r clones were grown in SGW without antibiotic for 24 h (Fig. 4A). Finally, the cell cultures were plated on SGW plates containing 5-FU (100 μg/mL). Colonies (Gen^s) grown on 5-FU plates were selected and verified for markerless deletion of Δ*narG* by PCR (Fig. 4B). We screened 30 5-FU^r clones, and five were confirmed as MD mutants via PCR amplification. Growth phenotypes of the PCR-verified Δ*narG* markerless strains were tested in anoxic SGW with nitrate as the electron acceptor. As expected, markerless deletion mutants of *narG* showed no growth while the host strains could grow in anoxic SGW with nitrate as the electron acceptor, demonstrating the loss of function of *narG* in these Δ*narG* markerless mutants (Fig. 5).

DISCUSSION

As more *Rhodanobacter* strains continue to be isolated and sequenced, there is an increasing need for a mutagenesis system to interrogate the molecular mechanisms underlying their tolerance to low pH and high concentrations of heavy metals. Deletion of target genes with a two-step integration and excision strategy has been favored in microbial functional genomics studies due to the advantage of no residual marker in the genome and limited polar effects. The choice of a counterselection marker is crucial for a markerless deletion strategy (21, 22). 5-FU resistance, derived from the deletion of the uracil PRTase gene *upp*, has been successfully used as a counterselectable marker in both Gram-positive and Gram-negative bacteria, such as *Bacillus subtilis* (23), *Lactobacillus acidophilus* (24), *Desulfovibrio vulgaris* (20), and *Enterococcus faecalis* (25). Given the presence of the uracil PRTase gene *upp* in *R. denitrificans* strains and the sensitivity of wild-type strains to 5-FU, Δ*upp*-derived 5-FU resistance was chosen as a counterselection marker in *R. denitrificans* in this study (see Fig. S7 and S9 in the supplemental material).

Despite the successful development of a markerless deletion mutagenesis system in two *Rhodanobacter* strains, the recombination efficiency was relatively low, and special attention is required for a few aspects. First, a defined medium such as SGW should be used in the counterselection step as the sensitivity of *Rhodanobacter* FW104 R3/R5

to 5-FU was not as strict as that of other bacteria such as *Desulfovibrio magneticus* or *Desulfovibrio vulgaris* (20, 26) (Fig. S7 and S9). When relatively richer R2A plates supplemented with 5-FU were used, more false-positive (i.e., wild-type) colonies were obtained. To increase the true-positive rate, we recommend selecting the larger colonies for PCR verification at the counterselection step. Larger colony sizes suggest better growth and higher resistance to 5-FU, which are consistent with the growth experiment showing a higher growth rate and higher maximum biomass in Δ*upp* strains than the wild-type strains (Fig. S7). Second, a two-step recombination strategy is required to ensure the occurrence of double-recombination events for generation of marker exchange (ME, Fig. 3) or markerless (MD, Fig. 2 and 4) mutants. In the first-step recombination, a single-crossover event allows the integration of the entire vector into the chromosome, using an antibiotic resistance gene (Gen^r in Fig. 2 and Kan^r Gen^r in Fig. 3 and 4) as selection. Then in the second-step recombination, these antibiotic-resistant clones were grown for 24 h without selection pressure, which allows for the occurrence of a second recombination event whereby the plasmid is excised. This results in either the creation of the desired mutation or reversion to wild type (see steps outlined in Fig. 2 to 4). To overcome some of the present limitations, Cas9/12-based genome editing might be an alternative approach in *Rhodanobacter* strains. However, the Cas9/12 approach also faces some challenges. For instance, finding suitable promoters to drive the expression of Cas protein and guide RNA may be arduous.

In summary, we demonstrated the development and application of an in-frame deletion mutagenesis approach using Δ*upp*-derived 5-FU resistance as a counterselection marker in two *R. denitrificans* strains. This method marks a crucial step in advancing *Rhodanobacter* as a model denitrifying bacterium for the study of denitrification in groundwater ecosystems and diverse molecular mechanisms of low-pH resistance. To our knowledge, this is the first report regarding the development of a targeted mutagenesis system in *Rhodanobacter* species. With the developed genetic manipulation approach in *Rhodanobacter*, further studies exploring the denitrification process in groundwater, pathways of nitrogen cycling, and heavy-metal turnover as well as many other longstanding questions are now possible. Our future studies aim to answer the following questions: why are *Rhodanobacter* species dominant in the low-pH and heavy metal-contaminated environment, and what are the key genes/proteins regulating and controlling uranium resistance and reduction? To improve the efficiency of the developed approach, deletion of certain RM genes or modification of the marker exchange or markerless vectors is needed. Finally, the in-frame deletion mutagenesis approach can be extended to other species of *Rhodanobacter*.

MATERIALS AND METHODS

Bacterial strains and plasmid construction. Strains, plasmids, and primers used in this study are listed in Tables 3 and 4. The genome sequences of FW104-R3 and FW104-R5 are available in the NCBI WGS database under accession numbers CP088921 and CP088980, respectively (19). For construction of pMD-upp, the pUC-19 ori, gentamicin resistance gene, and up/downstream homologous arms were amplified from pUC-19 (Invitrogen, Waltham, MA), pPROBE-GT (Addgene, Watertown, MA), and genomic DNA of wild-type *Rhodanobacter* FW104-R3 (FW104-R3 and FW104-R5 share the same homologous arm sequences), respectively. For construction of pME-narG, the *upp* gene including ~200 bp upstream and up/downstream homologous regions were amplified from genomic DNA of wild-type *Rhodanobacter* FW104-R3. The kanamycin resistance gene was amplified from pMO728 (20). The Ori^{pUC19}::Gen^r cassette was amplified from pMD-upp. The up/downstream homologous regions and the Ori^{pUC19}::Gen^r cassette in pMD-narG were the same as those of pME-narG. These fragments were assembled using Gibson assembly (New England Biolabs [NEB], Ipswich, MA) as described previously (27). The assembled product was transformed into *E. coli* for proliferation and selected by antibiotic screening. Plasmid sequences were verified via Sanger sequencing at the Oklahoma Medical Research Foundation. The barcoded Tn*5* transposon delivery vector pKMW7 was described previously (28).

Media and culture conditions. The *E. coli* DH5α strain (Invitrogen) was used for cloning and grown at 37°C in LB with either 15 μg/mL gentamicin or 50 μg/mL kanamycin. R2A (BD Bioscience, Franklin Lakes, NJ), TSA (BD Bioscience, Franklin Lakes, NJ), and SGW (6) media (the SGW medium was modified with the addition of trace minerals and vitamins [20]) with different pH ranges were used for optimization of growth conditions for all *Rhodanobacter* strains. For establishing the transformation protocol, SGW (pH 6.5) and R2A (pH 7.2) were also used. SGW medium (pH 6.5) was used for construction of marker replacement mutants and markerless deletion mutants. Solid SGW medium with 1.2% (wt/vol)

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
E. coli DH5α	Cloning strain	NEB (catalog no. C2987I)
DSM23569 (2APBS1)	Wild-type strain, isolated from FW107 well	DSMZ
DSM24678 (116-2)	Wild-type strain, isolated from contaminated area 3	DSMZ
FW104-10F02	Wild-type strain, isolated from FW104 well	This study
FW104-R3	Wild-type strain, isolated from FW104 well	This study
FW104-R5	Wild-type strain, isolated from FW104 well	This study
FW104-T7	Wild-type strain, isolated from FW104 well	This study
FW104-R8	Wild-type strain, isolated from FW104 well	This study
FW510-T8	Wild-type strain, isolated from FW510 well	This study
FW510-R10	Wild-type strain, isolated from FW510 well	This study
FW510-R12	Wild-type strain, isolated from FW510 well	This study
R3_Δ*upp*	Δ*upp* in FW104-R3	This study
R5_Δ*upp*	Δ*upp* in FW104-R5	This study
R3 Δ*upp* Δ*narG*::kan-upp	Δ*upp* and Δ*narG* in FW104-R3 (marker exchange)	This study
R5 Δ*upp* Δ*narG*::kan-upp	Δ*upp* and Δ*narG* in FW104-R5 (marker exchange)	This study
R3_Δ*upp*_Δ*narG*(MD)	Δ*upp* and Δ*narG* in FW104-R3 (markerless deletion)	This study
R5_Δ*upp*_Δ*narG*(MD)	Δ*upp* and Δ*narG* in FW104-R5 (markerless deletion)	This study
Plasmids		
pUC19	Amp^r	Invitrogen (catalog no. 18265017)
pPROBE-GT	Kan^r	Addgene
pBBR1MCS-5	Gen^r	Addgene
pMO728	Kan^r	19
pMD-upp	*upp* gene deletion vector; Gen^r	This study
pME-narG	*narG* gene exchange vector; Gen^r Kan^r	This study
pMD-narG	*narG* gene deletion vector; Gen^r	This study
Tn*5* transposon delivery vector pKMW7	Kan^r	28

Bacto agar (VWR, Radnor, PA) and corresponding antibiotics or 5-FU was used for plating and selection during mutant generation steps.

Preparation of competent cells and electroporation. The plasmid used for transformation was purified using the DNA Clean and Concentrator kit (Zymo Research, Irvine, CA). To prepare competent cells, glycerol stocks (150 μL) were inoculated into 3 mL SGW (pH 6.5) and incubated at 30°C with 200-rpm shaking overnight. The revived culture was transferred into 22 mL SGW (pH 6.5) and grown to mid-log phase (optical density at 600 nm of ~0.5). Then, the cell cultures were collected by centrifugation at 4,500 $\times$ *g* for 10 min and washed once with ice-cold electroporation buffer {30 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, pH 6.5}. The washed cells were resuspended in the electroporation buffer and kept on ice. For each transformation, a 50-μL cell suspension was mixed with 1.0 μg of plasmid DNA and 1 μL type I

TABLE 4 Primers used in this study

Primer name	Sequence, 5′–3′	Purpose
MD_upp_1	GCCTTTTGCTGGCCTTTTGCTCACATCCGCAGGTGATGGCGAAC	pMD-upp construction
MD_upp_2	TCGCGGCTGGTATCGGGGGCGGCTGTCTTCCGGGCA	pMD-upp construction
MD_upp_3	GGAAGACAGCCGCCCCCGATACCAGCCGCGAAGGA	pMD-upp construction
MD_upp_4	ATATTATACGCAAGGCGACAAGGTGCGACGCTGGGCATCGTGGTCG	pMD-upp construction
pUC-F	AGCTTTTCGCCCACGGCCTTGATGATCTGTCAGACCAAGTTTACTC	pMD-upp construction
pUC-R	ATGTGAGCAAAAGGCCAGCAAAAGGC	pMD-upp/pME-narG/pMD-narG construction
GenR-F	GCACCTTGTCGCCTTGCGTATAATAT	pMD-upp/pME-narG/pMD-narG construction
GenR-R	ATCATCAAGGCCGTGGGCGAAAAGCT	pMD-upp construction
ME_narG_1	GCCTTTTGCTGGCCTTTTGCTCACATAGGATGCGCAGGTGCGCGAAC	pME-narG/pMD-narG construction
ME_narG_2	CCCAGCTGGCAATTCCGGACACCTGTGCTGGCATCGCGA	pME-narG construction
ME_narG_3	AGCCCGTCGCGGCTCGCCTTCGGGCGCACGCTACCTGTC	pME-narG/pMD-narG construction
ME_narG_4	ATATTATACGCAAGGCGACAAGGTGCCTGGCGATGCCCTTGAAGATGT	pME-narG/pMD-narG construction
KanaNterm	CCGGAATTGCCAGCTGGG	pME-narG/pMD-narG construction
KanaCterm	CCCAGAGTCCCGCTCAGAAGAACTCG	pME-narG/pMD-narG construction
UppF	CGAGTTCTTCTGAGCGGGACTCTGGGTACCGCCGGCTCCTGTCGC	pME-narG/pMD-narG construction
UppR	AGGCGAGCCGCGACGGGCT	pME-narG/pMD-narG construction
MD_narG_2	GTAGCGTGCGCCCGAACACCTGTGCTGGCATCGCGA	pMD-narG construction
DUPP_F	TGCCACTACTTCGTCAACGG	Verification of deletion of *upp*
DUPP_R	TGGCGGATTTCCTCAAGCTC	Verification of deletion of *upp*

restriction inhibitor (Lucigen Corporation, Middleton, WI). The cells were electroporated in 1-mm-gap electroporation cuvettes (BTX, Holliston, MA) with an Eporator electroporator (Eppendorf, Hamburg, Germany). The voltage was 1.75 kV (1.25 kV was also used for the transformation test). The electroporated cells were transferred to 1 mL of SGW (pH 6.5) and incubated at 30°C with shaking. After recovery for about 20 h, the cells were collected by centrifugation at 5,000 × *g* at room temperature, and the cell pellet was spread on SGW (pH 6.5) agar plates supplemented with corresponding antibiotics (gentamicin, 15 μg/mL, and kanamycin, 50 μg/mL). The plates were incubated at 30°C for 48 to 72 h.

Generation of Δ*upp* host strains. The markerless deletion vector pMD-upp harboring the pUC-19 ori, gentamicin resistance gene, and up/downstream homologous arms of the *upp* gene was electroporated into the competent cells of wild-type *R. denitrificans* strains FW104-R3 and FW104-R5. After about 20 h of cell recovery in SGW (pH 6.5) without selection pressure, the diluted (1,000×) cell cultures were spread on SGW (pH 6.5) agar plates with 15 μg/mL gentamicin. Isolated colonies were screened using colony PCR with primers MD_upp_1 and MD_upp_4 to verify the integration of the vector in the chromosome through a single recombination event. Colonies with the expected PCR amplicon size were inoculated into SGW (pH 6.5) liquid medium for one more round of growth for about 24 h and then spread on SGW (pH 6.5) agar plates with 100 μg/mL 5-FU to select 5-FU^r clones. The in-frame deletion of the *upp* gene through a second crossover recombination event was verified using PCR using primers MD_upp_1 and MD_upp_4 and the expected PCR amplicon sizes.

Generation of Δ*narG* marker exchange mutants. The marker replacement (ME) vector pME-narG (Fig. 3A) was electroporated into the host strains R3_Δ*upp* and R5_Δ*upp* as described above. After electroporation of the ME vector, the recovered cells were spread on SGW (pH 6.5) agar plates containing 50 μg/mL kanamycin followed by a second round of screening on SGW plates containing 15 μg/mL gentamicin. The resulting antibiotic-resistant colonies were verified by colony PCR using primers ME_narG_1 and ME_narG_4. The colonies containing the integrated ME vector sequences were transferred into fresh SGW (pH 6.5) medium without antibiotics and incubated for about 24 h with shaking. The cell cultures were then spread on SGW (pH 6 0.5) agar plates with 50 μg/mL kanamycin. Individual colonies were selected and tested on gentamicin plates again. The Kan^r and Gen^s colonies were chosen and further verified by colony PCR using primers ME_narG_1 and ME_narG_4.

Generation of Δ*narG* markerless mutants. Construction of Δ*narG* markerless mutants was similar to construction of Δ*narG* marker exchange mutants with two steps. The markerless deletion (MD) vector pMD-narG was constructed in a similar way as that of pME-narG except there was no Kan^r-upp between the upstream and downstream homologous arms. In the first step, pMD-narG was electroporated into ME mutant R3 Δ*upp* Δ*narG*::kan-upp or R5 Δ*upp* Δ*narG*::kan-upp and the transformants were selected on SGW (pH 6.5) agar plates with 15 μg/mL gentamicin. Colony PCR with primers MD_narG_1 and MD_narG_4 was conducted to verify the integration of the vector in the chromosomes of the Gen^r clones. In a second step, the Gen^r clones from the first step were grown for about 24 h without antibiotic in SGW (pH 6.5) to allow the occurrence of the second recombination event, and the diluted (1,000×) cell cultures were plated on SGW (pH 6.5) agar plates with 150 μg/mL 5-FU. Colony PCR using primers MD_narG_1 and MD_narG_4 of the 5-FU^r colonies was conducted to verify that a true markerless deletion of *narG* was achieved.

Characterization of growth phenotypes of Δ*upp* and Δ*upp* Δ*narG* mutants. The growth curves of Δ*upp* mutant and wild-type strains were determined using a Bioscreen C (Growth Curves Ab Ltd., Helsinki, Finland) with four replicates per strain. The growth rates were calculated as previously reported (29). The wild-type strains FW104-R3 and FW104-R5 and Δ*upp* mutants were grown in SGW (pH 6.5) to mid-log phase and then streaked on SGW (pH 6.5) agar plates containing 50, 100, or 150 μg/mL 5-FU. For Δ*upp* Δ*narG* markerless strains, the host strains and Δ*narG* markerless strains were revived in SGW (pH 6.5) medium. The revived cultures (100 μL) were inoculated into 10 mL anoxic SGW (pH 6.5) medium containing 50 mM nitrate as an electron acceptor and glucose (5 g/L). Titanium citrate (30) was used as a reductant to remove any remaining oxygen. The growth of the Δ*upp* host strains and Δ*narG* markerless strains was measured using a spectrophotometer (Spectronic 20D+; Thermo Fisher, MA) at an optical density of 600 nm as described previously (27).

Data availability. All data are present in the article. The plasmids and primers used for mutagenesis are present in Tables 3 and 4, respectively. The genome sequences of FW104-R3 and FW104-R5 are available in the NCBI WGS database under accession numbers CP088921 and CP088980, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.1 MB.

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A.Z. and J.Z. developed the original concepts. A.Z. and X.T. designed the experiments. The *Rhodanobacter* strains were provided by R.C. All experiments were done by A.Z., X.T., M.L.K., J.L., M.P., and Y.L. X.T. and A.Z. wrote the paper. M.L.K., J.P.M., A.P.A., A.M.D., and J.Z.

edited the manuscript. All authors were given the opportunity to review the results and comment on the manuscript.

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