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Development of a Genetic System for *Geobacter sulfurreducens*

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Members of the genus *Geobacter* are the dominant metal-reducing microorganisms in a variety of anaerobic subsurface environments and have been shown to be involved in the bioremediation of both organic and metal contaminants. To facilitate the study of the physiology of these organisms, a genetic system was developed for *Geobacter sulfurreducens*. The antibiotic sensitivity of this organism was characterized, and optimal conditions for plating it at high efficiency were established. A protocol for the introduction of foreign DNA into *G. sulfurreducens* by electroporation was also developed. Two classes of broad-host-range vectors, IncQ and pBBR1, were found to be capable of replication in *G. sulfurreducens*. In particular, the IncQ plasmid pCD342 was found to be a suitable expression vector for this organism. When the information and novel methods described above were utilized, the *nifD* gene of *G. sulfurreducens* was disrupted by the single-step gene replacement method. Insertional mutagenesis of this key gene in the nitrogen fixation pathway impaired the ability of *G. sulfurreducens* to grow in medium lacking a source of fixed nitrogen. Expression of the *nifD* gene in *trans* complemented this phenotype. This paper constitutes the first report of genetic manipulation of a member of the *Geobacter* genus.

Dissimilatory metal-reducing microorganisms play an important role in the natural cycling of organic matter and minerals in aquatic sediments, submerged soils, and subsurface environments and can be important agents for the bioremediation of both organic and metal contamination (13–15). Molecular analyses (16S ribosomal DNA) have revealed that dissimilatory metal-reducing microorganisms in the genus *Geobacter* are prominent members of the microbial community in a diversity of environments in which dissimilatory metal reduction is either naturally occurring or artificially stimulated (22, 28). *Geobacter* species are obligate anaerobes belonging to the delta subdivision of the *Proteobacteria*. These organisms have the ability to completely oxidize organic compounds to carbon dioxide with either humic substances or Fe(III) as the sole electron acceptor (15). Other metals which can serve as electron acceptors for *Geobacter* species include Mn(IV), U(IV), Co(III), and Tc(VII). Several *Geobacter* species can also reduce nitrate and fumarate. The organic compounds oxidized by *Geobacter* species invariably include acetate and other short-chain fatty acids. In addition, some *Geobacter* species are capable of completely oxidizing monoaromatic compounds such as benzoate, phenol, *p*-cresol, and toluene to carbon dioxide with Fe(III) as the electron acceptor.

Little is known about the biochemical pathways that couple the oxidation of organic compounds to the reduction of metals in *Geobacter* species or about the regulation of these processes. Because many electron carriers will nonspecifically reduce metals and humic substances in vitro, it has been difficult to use biochemical studies to determine which of the numerous redox active proteins present in *Geobacter* species are actually involved in the reduction of metals and humic substances in vivo. It may therefore be easier to deduce the physiological roles of redox active proteins and enzyme complexes via a genetic approach—gene disruption followed by phenotypic analysis. Until now, the lack of a genetic system for *Geobacter* species has prevented the application of this type of approach to the study of the physiology of these organisms.

Here we report the development of a genetic system for *Geobacter sulfurreducens*. *G. sulfurreducens*, which was isolated from hydrocarbon-contaminated soil (7), has all of the important metabolic features of *Geobacter* species, including the ability to oxidize monoaromatic compounds (15). Furthermore, *G. sulfurreducens* also has the capacity to grow with fumarate serving as the sole electron acceptor, a property which is essential for the generation of mutants that are defective in the transfer of electrons to metals and humic substances.

Preliminary studies have suggested that *G. sulfurreducens* might have genes for nitrogen fixation (4). The ability to fix nitrogen may be required for *Geobacter* to compete successfully in petroleum-contaminated subsurface environments which are carbon rich but contain little fixed nitrogen (4). Methods for genetically manipulating *G. sulfurreducens* were developed as part of a study assessing the capacity of this organism to fix nitrogen. In this study, the targeted disruption of a *G. sulfurreducens* homolog of the *nifD* gene, a gene required for nitrogen fixation by other microorganisms (9), was found to eliminate the ability of *G. sulfurreducens* to grow in a medium devoid of fixed nitrogen. The ability of *G. sulfurreducens* to grow in this medium was restored when a functional copy of the gene was reintroduced in *trans*. These results indicate that *G. sulfurreducens* fixes nitrogen in a manner similar to that of other nitrogen-fixing microorganisms. The genetic techniques described herein should be applicable to the study of other aspects of *G. sulfurreducens* physiology and should make it possible to take full advantage of the information present in the forthcoming sequence of the genome of this environmentally significant organism.

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TABLE 1. Strains and plasmids

Species or plasmid	Strain or replicon(s); host range	Genotype or markers; characteristics and uses	Source or reference(s)
E. coli	DH5α	*supE44* Δ*lacU169* (ϕ *80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	5, 11
	JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} lacZΔ*M15*]	31
G. sulfurreducens	DL1	Wild type, derived from single colony isolated on an NBAFYE plate	7
	DL1/pCD354	DL1 containing pCD354	This work
	DL2A	*nifD1::kan*	This work
	DL2B	*nifD1::kan*	This work
	DL2C	*nifD1::kan*	This work
	DL2D	*nifD1::kan*	This work
	DL2D/pCDSnifD	DL2 containing pCDS*nifD*	This work
Plasmids			
pBBR1MCS-2	ND,[a] broad	Kan^r; unstable in *G. sulfurreducens*; source of kanamycin resistance cassette for *pBRnif::kan*	12
pBR322	pMB1; *E. coli*	Amp^r Tet^r; suicide vector in *G. sulfurreducens*	6
pBR*nif*	pMB1; *E. coli*	Amp^r; *G. sulfurreducens nifH*/*nifD* PCR fragment cloned into pBR322(Δ*Eco*RI-*Bam*HI); intermediate in the construction of pBR*nif::kan*	This work
pBR*nif::kan*	pMB1; *E. coli*	Amp^r Kan^r; kanamycin resistance cassette cloned into *Eco*RV site of pBR*nif*; suicide vector for construction of *nifD1::kan* mutants in *G. sulfurreducens*	This work
pCD342	IncQ; broad	Kan^r; expression vector; suitable for use in *G. sulfurreducens*	This work
pCD354	IncQ; broad	Kan^r; gfp_{mut2} expression vector; suitable for use in *G. sulfurreducens*	This work
pCD*nifD*	IncQ; broad	Kan^r; *G. sulfurreducens nifD* coding sequence cloned into pCD342 (Δ*Hin*dIII-*Eco*RI); intermediate in the construction of pCDS*nifD*	This work
pCDS*nifD*	IncQ; broad	Str^r; streptomycin resistance cassette cloned into pCD*nifD* (Δ*Bst*BI-*Bgl*II); *nifD* expression vector for *G. sulfurreducens nifD1::kan* mutants	This work
pBMK7	pMB1, pBG1; *E. coli*, *Desulfovibrio* spp.	Kan^r; *E. coli* and *Desulfovibrio* sp. shuttle vector; suicide vector in *G. sulfurreducens*	23

[a] ND, the incompatibility group of pBBR1MCS-2 has not been defined (2).

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and bacterial strains, *G. sulfurreducens* (ATCC 51573) and *Escherichia coli,* that were used in this study are listed and described in Table 1.

DNA manipulations and plasmid construction. Total *G. sulfurreducens* genomic DNA was prepared using the Genome DNA Kit (Bio 101, Inc., Carlsbad, Calif.). Primers for amplification of DNA fragments from the *G. sulfurreducens* chromosome were designed using sequence data obtained from The Institute for Genomic Research website at http://www.tigr.org. PCR amplification was performed using Ampli*Taq* DNA polymerase (Roche Molecular Systems, Branchburg, N.J.) according to the manufacturer's instructions. Plasmid DNA was purified from *E. coli* and *G. sulfurreducens* strains using the Qiagen midi- or miniplasmid purification kits (Qiagen Inc., Valencia, Calif.). DNA samples for electrotransformation were subjected to a second ethanol precipitation and 70% ethanol wash followed by resuspension in 0.5× TE buffer (0.5 mM Tris HCl [pH 8.0], 0.5 mM EDTA) in order to reduce their salt concentration and prevent arcing.

The suicide vector, pBR*nif::kan*, was constructed for the targeted disruption of the *G. sulfurreducens nifD* gene. A 1.1-kb fragment consisting of the first ~0.9 kb of the *nifD* coding region and ~0.2 kb of upstream sequence was amplified from the *G. sulfurreducens* chromosome using the primers prDL1 (**CCCCGCTGGAG ATGGAAGAGC**) and prDL2 (GGC*GGATCC***AGCCAGGGGATGCC**). In primer sequences, restriction sites are italicized and annealing nucleotides are indicated in boldface. This fragment was digested with *Eco*RI (cleaves 18 bp downstream of prDL1) and *Bam*HI (cleaves prDL2) and was inserted into the corresponding sites of pBR322 (6), resulting in the intermediate construct pBR*nif*. A kanamycin resistance cassette was amplified from pBBR1MCS-2 (12) with the primers prDL3 (CCC*GGTAACC***GGATGAATGTCAGC**) and prDL4 (CCC*GATATC***GC GGTGGAATCG**). The final suicide vector, pBR*nif::kan* (Fig. 2B), was constructed by inserting this cassette into a unique *Eco*RV site within pBR*nif* (at position 373 of the *nifD* coding sequence).

The *nifD* expression vector pCDS*nifD* was constructed in order to complement the *nifD1::kan* phenotype. The *nifD* coding sequence was amplified from *G. sulfurreducens* genomic DNA with the primers prDL5 (CCC*GGTACC***TGACAG GAGAATAC**) and prDL6 (CCC*AAGCTT***AAAAGCGGACTCCG**) and was inserted into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.). It was subsequently excised from this plasmid with *Eco*RI and *Hin*dIII and was inserted into the corresponding sites of the IncQ expression vector pCD342 (10). Because the resulting vector pCD*nifD* conferred resistance to kanamycin, it was not suitable for carrying out complementation studies in our *nifD1::kan* mutants. Therefore, the kanamycin resistance cassette of pCD*nifD* was excised with *Bgl*II and *Bst*BI and replaced with a streptomycin resistance cassette that had been amplified from pJRD215 (8) with the primers prDL7 (CCC*AGATCT***TTCTCATGTTTGA CAGC**) and prDL8 (CCC*TTCG*AA**TGAGATTGATGTGTTCC**).

Culturing conditions and growth media. The two *E. coli* strains listed in Table 1 were propagated according to established methods (26).

G. sulfurreducens strains were cultured at 30°C under strict anaerobic conditions as previously described (19). Plating and incubations on solid media were performed inside an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Mich.) containing a 7% H_2–10%CO_2–83% N_2 atmosphere heated to 30°C. *G. sulfurreducens* strains were propagated in two types of media (see compositions below): NBAF supplemented with 0.1% yeast extract and 1 mM cysteine (NBAFYE) and FWAFC. NBAFYE was used for general propagation and plating and for the preparation of electrocompetent cells, whereas FWAFC was used for the analysis of nitrogen fixation phenotypes. Antibiotics were added to cultures or plates as needed. During extended incubations of liquid cultures, fresh antibiotics were added every 48 h.

All media were dispensed into anaerobic pressure tubes or bottles with butyl rubber stoppers and, unless otherwise indicated, bubbled with an 80% N_2–20% CO_2 gas mixture to remove dissolved oxygen and obtain a final pH of ~6.7.

NBAF, a modified form of the medium described by Lovley et al. (16), contains 15 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor. Its composition per liter of deionized water is 0.42 g of KH_2PO_4, 0.22 g of K_2HPO_4, 0.2 g of NH_4Cl, 0.38 g of KCl, 0.36 g of NaCl, 0.04 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1.8 g of $NaHCO_3$, 0.5 g of Na_2CO_3, 2.04 g of $NaC_2H_3O_2 \cdot 3H_2O$, 6.4 g of $Na_2C_4H_4O_4$, 0.5 ml of 0.1% resazurin, 1.0 ml of 100 mM Na_2SeO_4, 10.0 ml of a vitamin solution (17), and 10.0 ml of NB trace mineral solution. The composition of the NB trace mineral solution per liter of deionized water is 2.14 g of nitriloacetic acid, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.3 g of $FeSO_4 \cdot 7H_2O$, 0.17 g of $CoCl_2 \cdot 6H_2O$, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 0.3 g of $CuCl_2 \cdot 2H_2O$, 0.005 g of $AlK(SO_4)_2 \cdot 12H_2O$, 0.005 g of H_3BO_3, 0.09 g of Na_2MoO_4, 0.11 g of $NiSO_4 \cdot 6H_2O$, and 0.2 g of $Na_2WO_4 \cdot 2H_2O$.

Liquid NBAFYE medium was prepared by adding appropriate volumes of sterile anoxic stock solutions of yeast extract and cysteine to NBAF medium

to achieve final concentrations of 0.1% (wt/vol) and 1 mM, respectively. If NBAFYE medium was to be used for manipulations within the anaerobic chamber, the final cysteine concentration was increased to 5 mM. Solid NBAFYE was prepared by amending NBAF medium lacking $CaCl_2$ and $MgSO_4$ with 15 g of purified agar/liter. Following sterilization, the molten medium was cooled to 50°C and appropriate volumes of anaerobic stock solutions of $CaCl_2$, $MgSO_4$, yeast extract, and cysteine were added to yield final concentrations of 0.004%, 0.01%, 0.1%, and 5 mM, respectively. If necessary, antibiotics (concentrations listed below) were also added at this time.

FWAFC is a modification of FWA-Fe(III) citrate medium (19), a freshwater minimal medium containing 20 mM acetate as the electron donor and 55.9 mM Fe(III) citrate as the electron acceptor. Its composition per liter of deionized water is 13.7 g of FeC_6O_7, 2.5 g of $NaHCO_3$, 0.25 g of NH_4Cl, 0.6 g of $NaH_3PO_4 \cdot H_2O$, 0.1 g of KCl, 1.0 ml of 100 mM Na_2SeO_4, 10.0 ml of a vitamin solution (17), and 10.0 ml of a trace mineral solution (17).

Ammonium- and nitrogen-free FWAFC medium was prepared by sparging and overlaying ammonium-free FWAFC medium with an 80% Ar–20% CO_2 gas mixture.

Antibiotics (200 μg of kanamycin, 400 μg of streptomycin, or 10 μg of chloramphenicol per ml) were added to liquid and solid medium as needed. All antibiotic stock solutions were sterile and anoxic (sparged and overlaid with an N_2 atmosphere).

Determination of plating efficiency. The cell density (cells/milliliter) of prestationary-phase liquid cultures was determined by staining cells with acridine orange and utilizing epifluorescence microscopy as previously described (19). The density of CFU in these cultures was determined by plating serial dilutions.

Preparation of electrocompetent cells. Electrocompetent *E. coli* was prepared according to Sambrook et al. (26). Electrocompetent *G. sulfurreducens* was prepared from cultures maintained in NBAFYE medium as described below.

All manipulations were carried out on ice in an anaerobic chamber, and any buffers used were ice-cold and anoxic. Four hundred milliliters of mid-log-phase cultures (optical density at 600 nm = 0.2 to 0.35; 9×10^7 to 1.8×10^8 cells/ml) was harvested by centrifugation at 4°C for 8 min at 4,300 × *g*. The cells were washed twice with 400 ml of electroporation buffer (1 mM HEPES [pH 7.0], 1 mM $MgCl_2$, and 175 mM sucrose) and resuspended in the same buffer at a final concentration of ~10^{11} cells/ml. Because *G. sulfurreducens* was found to be particularly susceptible to shearing, pipetting of cell suspensions was minimized and, when necessary, performed with large-bore pipette tips. An appropriate volume of a 60% dimethyl sulfoxide (DMSO)–40% electroporation buffer solution was added to the final cell suspension to achieve a final DMSO concentration of 10%. The resulting electrocompetent cells were either electroporated immediately or stored at −70°C for future use.

Electrotransformation procedures. All electrotransformations were performed in 0.15-cm-gap microelectroporation chambers using a Cell-Porator equipped with a Voltage Booster (Life Technologies Inc., Gaithersburg, Md.). Electrocompetent *E. coli* cells were transformed according to the manufacturer's instructions.

Electrocompetent *G. sulfurreducens* cells (25 μl), either freshly prepared or thawed on ice, were pulsed at 14.7 kV/cm for ~6 ms (resistance = 4 kΩ; capacitance = 25 μF). Immediately following electroporation, cells were washed into the bottom of the microelectroporation chamber with 1 ml of room temperature phosphate-buffered NBAF (NBAF medium containing 50 mM potassium phosphate instead of bicarbonate). The cells were then transferred to a prewarmed anaerobic pressure tube containing 9 ml of NBAFYE. The electroporated cells were allowed to recover for 5 h at 30°C, prior to plating onto the appropriate solid growth medium.

Assessment of plasmid stability under nonselective conditions. *G. sulfurreducens* was electrotransformed with either pBBR1MCS-2 or pJRD215 (Table 1) and plated onto selective medium (NBAFYE containing 200 μg of kanamycin/ml). The resulting kanamycin-resistant colonies were transferred to liquid selective medium until the cultures became turbid (3 to 6 days). Selective pressure was maintained throughout this incubation by adding fresh kanamycin to the cultures every 48 h. Twenty-four hours prior to the beginning of the experiment, cultures were diluted 10-fold into fresh selective medium. At time zero, the cultures were serially diluted into nonselective medium (NBAFYE) and were then plated onto both selective and nonselective media. The remainder of the various serial dilutions was incubated at 30°C. Every 48 h, a log-phase culture was selected from among the various dilutions for analysis and the process was repeated. The total number of CFU in the various cultures was determined on nonselective medium, and the percentage of kanamycin-resistant CFU was ascertained by comparison. The number of generations between time points was determined from the total CFU/milliliter using the following formula: log (final CFU per milliliter/initial CFU per milliliter)/$\log_2$.

Southern blotting. Following digestion of genomic DNA with the restriction enzyme *Eco*RV, Southern blot analysis was performed according to Sambrook et al. (26). Probes were labeled with [α-^{32}P]dCTP using the Multiprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, N.J.). [α-^{32}P]dCTP was obtained from New England Nuclear (Boston, Mass.).

RESULTS AND DISCUSSION

Growth on solid medium and characterization of antibiotic sensitivity. In order to be able to rapidly isolate clonal populations of *G. sulfurreducens*, conditions for high-efficiency plating onto solid growth medium were established. Simply solidifying the two liquid media for *G. sulfurreducens*, NBAF and FWAFC (see Materials and Methods), was not sufficient to achieve the rapid growth of *G. sulfurreducens* on plates. After considerable trial and error, it was found that supplementation of the solid form of NBAF medium with 0.1% yeast extract and a high concentration (5 mM) of the reductant cysteine yielded a solid medium, NBAFYE, on which both the growth rate and the plating efficiency of *G. sulfurreducens* were reproducibly high. Colonies were visible on solid NBAFYE medium after only 5 days of incubation at 30°C. In addition, when prestationary-phase liquid cultures with a density of $(3.25 \pm 0.54) \times 10^8$ cells/ml were plated onto this medium, $(2.85 \pm 0.48) \times 10^8$ CFU/ml were recovered (mean ± standard deviation; $n = 3$), demonstrating a high plating efficiency.

The growth of *G. sulfurreducens* on solid medium could be inhibited by a variety of commonly used antibiotics. Growth of 10^8 cells on solid medium could be inhibited by choramphenicol (10 μg/ml), nalidixic acid (10 μg/ml), tetracycline (10 μg/ml), kanamycin (200 μg/ml), spectinomycin (50 μg/ml), streptomycin (400 μg/ml), and ampicillin (400 μg/ml). The sensitivity of *G. sulfurreducens* to these antibiotics in liquid culture was similar (data not shown). These results indicated that it should be possible to select for the acquisition of multiple antibiotic resistance markers by *G. sulfurreducens*, a requirement for many genetic manipulations.

Development of an electrotransformation procedure. A protocol for the electrotransformation of *G. sulfurreducens* was developed by modifying existing protocols (21) to account for the unique properties of *G. sulfurreducens*. The preparation of electrocompetent cells typically involves extensive washing of cells derived from mid-log-phase cultures in a suitable low-ionic-strength electroporation buffer, followed by resuspension of the cells at high density (10^9 to 10^{11} cells/ml) in this buffer (21). In order to attain sufficient *G. sulfurreducens* biomass at mid-log phase, it was necessary to supplement the NBAF medium routinely used to culture the organism with 0.1% (wt/vol) yeast extract. When grown on this rich medium at 30°C, *G. sulfurreducens* had a generation time of ~4.5 h and reached a cell density of ~10^8 cells/ml at mid-log phase (data not shown). The composition of the electroporation buffer was found to be critical for maintaining the viability of *G. sulfurreducens* throughout the washing and electrotransformation procedures. The optimal composition of the electroporation buffer was found to be 1 mM HEPES (pH 7.0), 1 mM $MgCl_2$, and 175 mM sucrose. The addition of $MgCl_2$ to the electroporation buffer was absolutely required if *G. sulfurreducens* was to remain viable during washing and storage. In the absence of $MgCl_2$, 90% of the cells lysed prior to electroporation (data not shown). $MgCl_2$ is thought to prevent bacterial lysis by stabiliz-

ing the outer membrane (27). The concentration of sucrose was another crucial variable in the composition of the electroporation buffer. Electrotransformed *G. sulfurreducens* could not tolerate large changes in osmolarity upon dilution back into nonselective growth medium. To avoid such changes in osmotic pressure, 175 mM sucrose was added to the electroporation buffer such that its osmolarity was equivalent to that of NBAFYE medium. The addition of 10% DMSO to the final cell suspension permitted storage of electrocompetent *G. sulfurreducens* at −70°C with little or no loss in transformation efficiency. Glycerol was not a suitable cryopreservant due to the sensitivity of *G. sulfurreducens* to rapid changes in osmolarity.

Electroporation parameters were optimized by transforming *G. sulfurreducens* with the broad-host-range IncQ plasmid pJRD215 (Table 1). Electrotransformation of *G. sulfurreducens* with pJRD215 resulted in the growth of kanamycin-resistant colonies. The presence of pJRD215 in these colonies was confirmed by PCR (data not shown) and by digesting plasmid DNA purified from these colonies with restriction enzymes (Fig. 1A, lane 1). Electrotransformation was optimal when *G. sulfurreducens* was pulsed at 14.7 kV/cm with a time constant of approximately 6 ms. Under these conditions, $(1.98 \pm 0.32) \times 10^5$ transformants/μg of pJRD215 DNA were obtained. Electroporation lethality was roughly 59% ± 5.23%, and transformants constituted 1 out of $(1.19 \pm 0.43) \times 10^4$ CFU. (Data are means ± standard errors; $n = 11$). These data were within the range reported for other bacteria in which gene replacement has been successful (3, 23, 24, 29).

As shown in Table 2, transformation efficiency increased to $(2.96 \pm 1.08) \times 10^7$ transformants/μg (mean ± standard error; $n = 3$) when pJRD215 DNA was purified from *G. sulfurreducens.* This 100-fold increase in transformation efficiency suggests that *G. sulfurreducens* may possess a robust restriction system.

Identification of potential "suicide" and expression vectors. A variety of plasmids were tested for the ability to replicate in and confer antibiotic resistance upon *G. sulfurreducens* (Table 2). In addition to pJRD215, two other IncQ plasmids, pJRC2 and pCD342 (Table 1), were evaluated. The plasmid pJRC2 is identical to pJRD215 except that it confers resistance to chloramphenicol. pCD342 is an expression vector containing a polylinker which is preceded by the hybrid *tac-lac* promoter (*tac* and *lacUV5* promoters in tandem) and followed by the *rrnB* transcriptional terminator (1, 20). Electroporation of *G. sulfurreducens* with all three IncQ plasmids resulted in the growth of numerous antibiotic-resistant transformants (Table 2), indicating that all of these plasmids were capable of replication in *G. sulfurreducens.* In addition, all three of the IncQ plasmids could be purified from cultures of *G. sulfurreducens* in sufficient quantity to be identified by digestion with restriction enzymes (Fig. 1A), suggesting that IncQ plasmids are stable in this organism. In fact, pJRD215 has a half-life of ~60 gener-

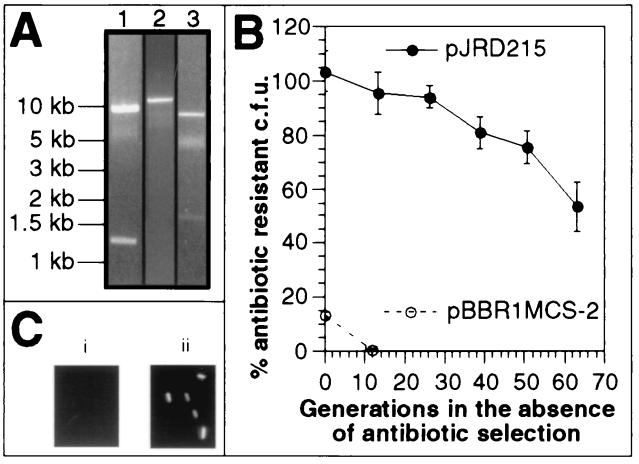

FIG. 1. Stability of IncQ and pBBR1 vectors in *G. sulfurreducens*. (A) Purification of IncQ plasmids from *G. sulfurreducens* transformants. Plasmid DNA was purified from *G. sulfurreducens* transformed with pJRD215 (lane 1), pJRC2 (lane 2), and pCD342 (lane 3) by alkaline lysis (26). Following purification, plasmid DNA was digested with the restriction enzymes *Bgl*II and *Hin*dIII. The sizes of the expected restriction fragments for the three plasmids are as follows: 9.1 and 1.2 kb for pJRD215; 10.4 kb for pJRC2 (23); and 8.0 and 1.7 kb for pCD342 (10). (B) Stability of pJRD215 and pBBR1MCS-2 in *G. sulfurreducens* under nonselective conditions. Assessment of the stability of these plasmids in the absence of antibiotic selection is described in detail in Materials and Methods. Data are means ± standard errors of four independent experiments. (C) Expression of green fluorescent protein from an IncQ expression vector in *G. sulfurreducens*. Green fluorescent protein expression was visualized by Nomarski microscopy using a fluorescein isothiocyanate filter. Panel i, DL1, wild-type *G. sulfurreducens*. Panel ii, DL1/pCD354, *G. sulfurreducens* transformed with pCD354 (10).

TABLE 2. Transformation of *G. sulfurreducens* with a variety of plasmids

Plasmid	Replicon(s)	Host specificity	Antibiotic resistance	No. of transformants[a] per μg of DNA	Stability (half-life in absence of antibiotic selection)
pJRD215	IncQ	Broad	Kan^r Str^r	$(1.98 \pm 0.32) \times 10^5$ (11) $(2.96 \pm 1.08) \times 10^7$ (3)[c]	~60 generations
pJRC2	IncQ	Broad	Chl^r Str^r	$(4.06 \pm 2.31) \times 10^3$ (3)	Not determined
pCD342	IncQ	Broad	Kan^r	$(1.24 \pm 0.37) \times 10^4$ (3)	Not determined
pBBR1MCS-2	ND[b]	Broad	Kan^r	$(2.79 \pm 0.41) \times 10^4$ (3)	<10 generations
pBMK7	pMB1, pBG1	*E. coli, Desulfovibrio* spp.	Kan^r	0 (5)	Not determined

[a] Data are means ± standard errors, and the number of experiments performed is indicated in parentheses. The number of transformants per microgram of DNA was determined by counting kanamycin-resistant colonies except in the case of pJRC2, in which chloramphenicol-resistant colonies were counted. Unless otherwise indicated, *G. sulfurreducens* was transformed with plasmid DNA purified from *E. coli* strain JM109 or DH5α. The stability of pJRD215 and pBBR1MCS-2 was determined as described in Materials and Methods.

[b] ND, the incompatibility group of pBBR1MCS-2 has not been defined (2).

[c] The number of transformants obtained when pJRD215 was purified from *G. sulfurreducens*.

ations in *G. sulfurreducens* in the absence of antibiotic selection (Table 2; Fig. 1B). Similarly, we were able to purify pCD342 from *G. sulfurreducens* (Fig. 1A, lane 3) after culturing a transformant for approximately 30 generations in the absence of antibiotic selection.

To determine whether pCD342 could serve as an expression vector for *G. sulfurreducens*, cells were transformed with pCD354 (10), a green fluorescent protein expression vector derived from pCD342. The green fluorescent protein was detected in the resulting transformants (Fig. 1C), indicating that pCD342 is indeed a suitable expression vector for *G. sulfurreducens*.

Due to their large size (≥ 10 kb) and low copy number, IncQ vectors are difficult to manipulate (8, 10). The pBBR1MCS series of broad-host-range expression vectors (12), in contrast, are fairly small (~5.3 kb) and have a higher copy number. In addition, they contain a variety of antibiotic resistance cassettes and possess an extensive multicloning site located within the *lacZ* alpha peptide (12). Kanamycin-resistant transformants were isolated following electroporation of *G. sulfurreducens* with pBBR1MCS-2 (Table 2), indicating that this class of plasmids is capable of replication in *G. sulfurreducens.* However, pBBR1MCS-2 was found to be unstable in *G. sulfurreducens* (Fig. 1B). Despite the rigorous maintenance of selective pressure during propagation of pBBR1MCS-2 in liquid culture, only 15% of the CFU in these cultures were able to grow on solid medium containing kanamycin. Furthermore, in the absence of continued selection, kanamycin resistance was lost within 12 generations. Thus, despite the fact that pBBR1MCS vectors can replicate in *G. sulfurreducens*, their instability renders them unsuitable for use as expression vectors.

In addition to the two types of broad-host-range plasmids mentioned above, one plasmid with limited host specificity, pBMK7 (23), was evaluated for the ability to replicate in *G. sulfurreducens.* The plasmid pBMK7 is a shuttle vector that contains two replicons: the pBM1 replicon, which functions in *E. coli,* and the pBG1 replicon, which functions in *Desulfovibrio* species. Members of the genus *Desulfovibrio*, like those of the *Geobacter* genus, are obligate anaerobes belonging to the delta subdivision of the *Proteobacteria*. However, electrotransformation of *G. sulfurreducens* with pBMK7 repeatedly failed to yield kanamycin-resistant transformants (Table 2). Thus, even though *Geobacter* and *Desulfovibrio* species are closely related, the pBG1 replicon did not appear to function in *G. sulfurreducens.* Furthermore, these results indicated that plasmids containing the pBG1 or pBM1 replicons acted as suicide vectors in *G. sulfurreducens* and could, therefore, be used to introduce mutations into its chromosome.

Mutagenesis of the *G. sulfurreducens* chromosome by gene replacement. The next step in the development of a genetic system for *G. sulfurreducens* was to determine whether the function of a specific gene could be eliminated by placing a mutation in the *G. sulfurreducens* chromosome. A gene suspected of being involved in nitrogen fixation by *G. sulfurreducens* was selected as the first target, because, as noted above, it is important to conclusively demonstrate that *G. sulfurreducens* has the capacity to fix nitrogen. In other microorganisms, nitrogen reduction is catalyzed by the nitrogenase enzyme complex, which is encoded by the highly conserved *nifH*, *nifD*, and *nifK* genes (9, 30). Because the nitrogenase complex functions specifically in the nitrogen fixation pathway, inactivation of the genes for any of its components yields a predictable and easily characterized phenotype, an inability to grow in medium lacking fixed nitrogen (9). By using the *nifD* genes of other organisms to probe the partial sequence of the *G. sulfurreducens* genome, a potential *G. sulfurreducens nifHDK* operon was identified. Studies were initiated to determine whether disruption of the putative *nifD* gene of *G. sulfurreducens* by the single-step gene replacement method (25) would affect the

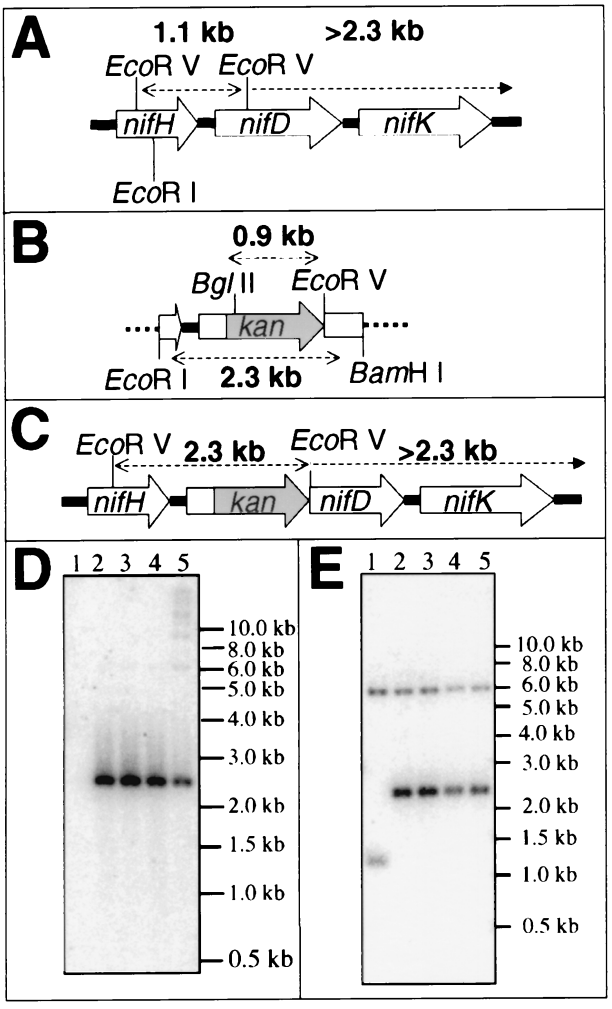

FIG. 2. Confirmation of *nifD* gene disruption by Southern blot analysis. (A) Restriction map of the *G. sulfurreducens nifHDK* operon. (B) Restriction map of pBR*nif::kan*. The vector pBR322 is indicated by thick dotted lines. (C) Restriction map of *G. sulfurreducens nifHDK* operon containing the *nifD1::kan* insertion mutation. (D) Southern blot of genomic DNA prepared from wild-type (lane 1) and *nifD1::kan* strains (lanes 2 to 5) of *G. sulfurreducens*. Genomic DNA was digested with the restriction enzyme *Eco*RV and probed with a *Bgl*II/*Eco*RV restriction fragment of the kanamycin resistance cassette of pBR*nif::kan*. Expected radiolabeled bands are as follows: lane 1, none; lanes 2 to 5, 2.3 kb. (E) Southern blot of genomic DNA prepared from wild-type (lane 1) and *nifD1::kan* (lanes 2 to 5) strains of *G. sulfurreducens*. Genomic DNA was digested with the restriction enzyme *Eco*RV, blotted, and then probed with a *Bam*HI/*Eco*RI restriction fragment of pBR*nif::kan*. Expected radiolabeled bands are as follows: lane 1, 1.1 and >2.3 kb; lanes 2 to 5, 2.3 and >2.3 kb. All restriction maps were based on sequence data obtained from http://www.tigr.org.

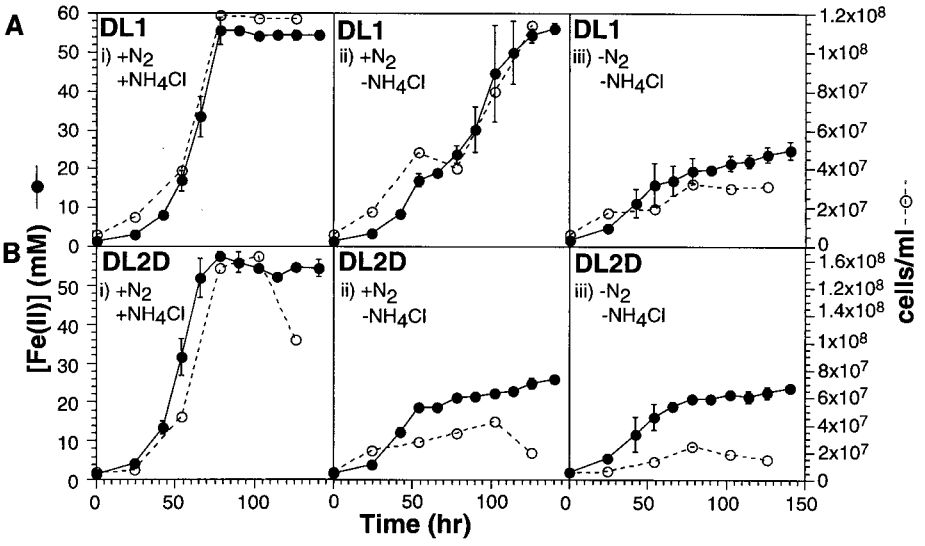

FIG. 3. Nitrogen fixation by wild-type (DL1) and *nifD1::kan* (DL2D) *G. sulfurreducens.* FWAFC cultures of strains DL1 and DL2D were washed three times with FWAFC medium lacking both N_2 and NH_4Cl (N_2 replaced by Ar) in order to obtain cell suspensions containing as little N_2 and NH_4Cl as possible. At time zero, the washed cells were inoculated into three types of FWAFC media: (i) FWAFC, (ii) ammonium-free FWAFC, and (iii) FWAFC lacking both N_2 and NH_4Cl at a final concentration of $\sim 6 \times 10^6$ cells/ml. Fe(II) concentration (in millimolars) and cell density (in cells/milliliter) were determined on 0.1-ml samples as previously described (18, 19). Fe(II) concentrations are means ± standard deviations of measurements obtained from three independent cultures, whereas cell densities were determined from one representative culture.

ability of *G. sulfurreducens* to grow in a medium devoid of fixed nitrogen.

In order to disrupt the putative *G. sulfurreducens nifD* gene, we constructed the suicide vector pBR*nif::kan* (Table 1; Fig. 2B), which contained a fragment of the *nifD* coding sequence disrupted by a kanamycin resistance cassette. To ensure that incorporation of the disrupted *nifD* coding sequence into the chromosome occurred via a double recombination event, the suicide vector was linearized with *Nru*I and *Sca*I prior to electroporation. The recovery period following electroporation was extended to ~18 h to allow ample time for recombination and expression of kanamycin resistance. This procedure resulted in the growth of 2.77×10^5 kanamycin-resistant colonies per μg of linear DNA. Eight of these kanamycin-resistant colonies were screened for the presence of the disrupted *nifD* (*nifD1::kan*) coding sequence by PCR, and all eight were found to contain the *nifD1::kan* insertion mutation (data not shown). These results indicated that recombination between linear DNA fragments and the *G. sulfurreducens* chromosome was a highly efficient process.

As shown in Fig. 2, Southern blot analysis confirmed both the presence and placement of the kanamycin resistance cassette within the 5′ end of the *nifD* gene in four of the eight *nifD1::kan* mutants described above, strains DL2A to -D. All four of these genetically identical mutants were unable to completely reduce the available Fe(III) in FWAFC medium lacking a source of fixed nitrogen (data not shown). This result suggested that disruption of the *nifD* gene had resulted in the expected phenotype, an inability to grow in the absence of exogenous fixed nitrogen. One of the four mutants, DL2D, was selected for a more detailed characterization of its phenotype (Fig. 3).

When strain DL2D and the wild-type strain DL1 were inoculated into FWAFC medium, they grew and reduced Fe(III) at similar rates (Fig. 3). When ammonium, the source of fixed nitrogen in the medium, was omitted, the wild-type strain exhibited a longer lag but eventually grew and reduced Fe(III) to levels comparable to those observed in ammonium-containing medium. The extended lag period most probably reflected the time required to induce the expression of nitrogen fixation genes. In contrast to the wild-type strain, the mutant strain DL2D failed to grow significantly in ammonium-free FWAFC medium and reduced only a small amount of the available Fe(III). In fact, the amount of growth and Fe(III) reduction by the mutant strain in ammonium-free medium was comparable to that in medium lacking both N_2 and ammonium.

The small amount of cell growth observed in medium devoid of both N_2 and ammonium (Fig. 3) can be attributed to the fact that residual ammonium may have been present in the washed starter cultures and that any cell lysis that occurred during washing would have resulted in the release of additional fixed nitrogen. The sustained low rate of Fe(III) reduction seen in this medium at the end of the experiment is probably due to the fact that *G. sulfurreducens* is still capable of reducing Fe(III) even when it is not actively growing.

Complementation of the *nifD1::kan* phenotype by in *trans* expression of the *nifD* gene. In order to demonstrate that the phenotype of mutant DL2D was in fact due to disruption of the *nifD* gene and not to an unexpected secondary mutation or to an unanticipated effect on downstream gene expression, the *nifD* expression vector pCDS*nifD* was introduced into DL2D by electroporation. To determine if expression of the *nifD* gene in *trans* complemented the *nifD1::kan* phenotype, the ability of DL2D/pCDS*nifD* to grow in the absence of fixed nitrogen was evaluated (Fig. 4). The extent of cell growth and iron reduction

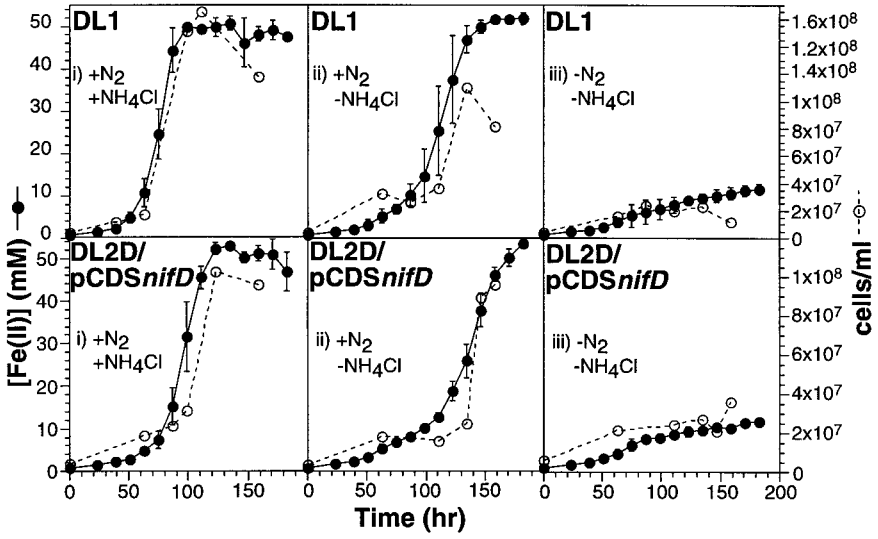

FIG. 4. Complementation of *nifD1::kan* phenotype by expression of *nifD* gene in *trans*. In order to select for the maintenance of pCDS*nifD*, strain DL2D/pCDS*nifD* was maintained in FWAFC medium containing 400 mg of streptomycin/ml until the beginning of the experiments. To obtain cell suspensions containing as little N_2, NH_4Cl, and streptomycin as possible, FWAFC cultures of strains DL1 (wild type) and DL2D/pCDS*nifD* were washed three times with FWAFC medium without both N_2 and NH_4Cl (N_2 replaced by Ar). At time zero, washed cells were inoculated into three types of FWAFC media: (i) FWAFC, (ii) ammonium-free FWAFC, and (iii) FWAFC lacking both N_2 and NH_4Cl at a final concentration of $\sim 3.6 \times 10^6$ cells/ml. Fe(II) concentration and cell density were determined on 0.1-ml samples as previously described (18, 19). Fe(II) concentrations are the means ± standard deviations of measurements obtained from three independent cultures, whereas cell densities were determined from one representative culture.

by both DL2D/pCDS*nifD* and the wild-type strain (DL1) was similar under all three conditions tested (Fig. 4): (i) in the presence of both gaseous and fixed nitrogen, (ii) in the presence of gaseous nitrogen alone, and (iii) in the absence of both gaseous and fixed nitrogen. Thus, expression of the *nifD* gene in *trans* restored the ability of the *nifD1::kan* mutant DL2D to grow in the absence of exogenous fixed nitrogen. In fact, DL2D/pCDS*nifD* can be routinely cultured in ammonium-free FWAFC medium (data not shown).

Although antibiotics, which constitute potential sources of fixed nitrogen, could not be used to select for the maintenance of pCDS*nifD* and the *nifD1::kan* mutation over the course of this experiment, PCR screening confirmed the presence of both the *nifD1::kan* mutation and pCDS*nifD* in strain DL2D/pCDS*nifD* at the end of the experiment (data not shown).

The results described above confirm that *G. sulfurreducens* has the ability to fix nitrogen. In addition, these results demonstrate that it is possible to complement mutations in *G. sulfurreducens*, a genetic technique required for properly evaluating the function of genes in this organism.

Concluding remarks. In summary, this study has demonstrated that *G. sulfurreducens* is a genetically tractable microorganism. It is now possible to introduce foreign DNA into *G. sulfurreducens* by electroporation, to place mutations at specific locations within its chromosome, and to express proteins from extrachromosomal elements. These novel genetic techniques coupled with the availability of the sequence of the *G. sulfurreducens* genome will enable the rapid study of the physiological function of multiple *G. sulfurreducens* genes. The finding that the physiology of *G. sulfurreducens* can be studied by genetic techniques is significant, because microorganisms closely related to *G. sulfurreducens* are prominent members of a variety of Fe(III)-reducing microbial communities (22, 28). Thus, studies on the physiology of *G. sulfurreducens*, using the techniques outlined here, are likely to provide insights into microbial processes occurring in the Fe(III) reduction zone of subsurface environments. Studies similar to those described here but focusing on mechanisms for the reduction of Fe(III) and contaminant metals by *G. sulfurreducens* are currently in progress.

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