Scarless Genome Editing and Stable Inducible Expression Vectors for Geobacter sulfurreducens

Chi Ho Chan, a Caleb E. Levar, a,b Lori Zacharoff, a,c Jonathan P.Badalamenti, a Daniel R. Bond a,b

BioTechnology Institute, a Department of Microbiology, a and Department of Biochemistry, Molecular Biology, and Biophysics, c University of Minnesota-Twin Cities, Saint Paul, Minnesota, USA

Metal reduction by members of the Geobacteraceae is encoded by multiple gene clusters, and the study of extracellular electron transfer often requires biofilm development on surfaces. Genetic tools that utilize polar antibiotic cassette insertions limit mutant construction and complementation. In addition, unstable plasmids create metabolic burdens that slow growth, and the presence of antibiotics such as kanamycin can interfere with the rate and extent of Geobacter biofilm growth. We report here genetic system improvements for the model anaerobic metal-reducing bacterium Geobacter sulfurreducens. A motile strain of G. sulfurreducens was constructed by precise removal of a transposon interrupting the fgrM flagellar regulator gene using SacB/sucrose counterselection, and Fe(III) citrate reduction was eliminated by deletion of the gene encoding the inner membrane cytochrome imcH. We also show that RK2-based plasmids were maintained in G. sulfurreducens for over 15 generations in the absence of antibiotic selection in contrast to unstable pBBR1 plasmids. Therefore, we engineered a series of new RK2 vectors containing native constitutive Geobacter promoters, and modified one of these promoters for VanR-dependent induction by the small aromatic carboxylic acid vanillate. Inducible plasmids fully complemented ΔimcH mutants for Fe(III) reduction, Mn(IV) oxide reduction, and growth on poised electrodes. A real-time, high-throughput Fe(III) citrate reduction assay is described that can screen numerous G. sulfurreducens strain constructs simultaneously and shows the sensitivity of imcH expression by the vanillate system. These tools will enable more sophisticated genetic studies in G. sulfurreducens without polar insertion effects or need for multiple antibiotics.

Methods to remove, replace, and control genes are instrumen-
tal to understanding bacterial physiology. Together with DNA synthesis and high-throughput sequencing, these tools enable synthetic reconstruction of pathways and design of biological circuitry (1). Many genetic approaches were first developed in fast-growing bacteria such as Escherichia coli, while genetic techniques able to interrogate the physiology of slower-growing anaerobic organisms are often more limited (2). In some cases, heterologous expression of foreign genes can help infer function in a genetically intractable organism (3), but model hosts can lack key biochemical processes or cofactors making functional expression challenging (4). Electron transfer to metals and electrodes by Geobacter sulfurreducens is an example of a complex respiratory strategy encoded in multigene loci throughout the chromosome, requiring cytochrome maturation, protein secretion, cell surface attachment, sensing, and motility (5–8). The genetic study of metal reduction will ultimately require deletion and reexpression of multiple genes in the native organism, under a variety of planktonic and long-term biofilm growth conditions.

A gene replacement protocol using electroporation of linear DNA fragments was first developed for G. sulfurreducens in 2001 (9), and insertion of antibiotic cassettes allowed for construction of multiple deletion mutants, such as the ΔomcB::cat ΔomcST::npII ΔomcE::aacC ΔomcZ::aadA quintuple deletion mutant. However, due to the finite number of resistance genes available in Geobacter, it is difficult to delete additional loci or complement such complex constructs (10). Conjugal plasmid transfer from an Escherichia coli donor strain, and transposon mutagenesis via nonreplicating plasmids is also feasible in G. sulfurreducens (9, 11). These tools accelerate mutant construction and discovery, but issues of transposon insertion polarity, growth inhibition due to use of multiple antibiotics, and unknown expression levels from promoters on plasmids limit complementation and interpretation of results (12, 13). A cre-lox recombination gene disruption strategy used in G. metallireducens and G. sulfurreducens removes the antibiotic cassette from the chromosome (14, 15). However, this method leaves a loxP scar sequence and creates multiple identical loxP sequences throughout the genome if additional deletions are constructed (16).

In this study, we implemented a SacB/sucrose counterselection strategy to generate scarless deletions in G. sulfurreducens. We also tested the stability and the effect of commonly used broad-host-range plasmids on growth using soluble, insoluble, and poised electrode electron acceptors. New vectors based on the RK2 origin of replication with native constitutive and engineered inducible promoters were constructed for controlled gene expression in G. sulfurreducens using different electron acceptors. To accelerate the screening of constructs and promoters, we describe a real-time, high-throughput Fe(III) citrate reduction assay to measure the
response of the engineered promoter system. These new tools make it possible to construct multigene deletions, promoter modifications, in-frame fusions, and inducible genetic circuitry in Geobacter sulfurreducens.

MATERIALS AND METHODS

Growth and medium conditions. All strains and plasmids used in the present study are listed in Table 1. Geobacter sulfurreducens strains were cultured at 30°C in anoxic liquid basal minimal medium with 20 mM acetate as the electron donor and 40 mM fumarate, 50 mM ferric citrate, cultured at 30°C in anoxic liquid basal minimal medium with 20 mM Mn(IV) oxide as the electron acceptor in sealed anaerobic tubes containing Fe(III) citrate or Mn(IV) oxide, a nonchelated mineral mix containing Fe(III). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV).

MATERIALS AND METHODS

Growth and medium conditions. All strains and plasmids used in the present study are listed in Table 1. Geobacter sulfurreducens strains were cultured at 30°C in anoxic liquid basal minimal medium with 20 mM acetate as the electron donor and 40 mM fumarate, 50 mM ferric citrate, cultured at 30°C in anoxic liquid basal minimal medium with 20 mM Mn(IV) oxide as the electron acceptor in sealed anaerobic tubes containing Fe(III) citrate or Mn(IV) oxide, a nonchelated mineral mix containing Fe(III). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV).

response of the engineered promoter system. These new tools make it possible to construct multigene deletions, promoter modifications, in-frame fusions, and inducible genetic circuitry in Geobacter sulfurreducens.

MATERIALS AND METHODS

Growth and medium conditions. All strains and plasmids used in the present study are listed in Table 1. Geobacter sulfurreducens strains were cultured at 30°C in anoxic liquid basal minimal medium with 20 mM acetate as the electron donor and 40 mM fumarate, 50 mM ferric citrate, cultured at 30°C in anoxic liquid basal minimal medium with 20 mM Mn(IV) oxide as the electron acceptor in sealed anaerobic tubes containing Fe(III) citrate or Mn(IV) oxide, a nonchelated mineral mix containing Fe(III). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV). A 1:5 dilution in 0.5 N HCl was performed prior to measuring the Mn(IV).
fumarate medium described above to an OD_{600} of 0.5 from single colonies obtained from a fresh plate in an anaerobic glove box. Colonies were grown in 1 ml of medium and then transferred 1:10 into 10 ml of fresh acetate-fumarate medium. As this culture reached an OD_{600} of 0.5, electrode bioreactor growth was initiated to achieve a density of 0.125 OD_{600} in the bioreactor. Bioreactor medium was composed of 20 mM acetate basal medium with 2.9 g/liter NaCl added for osmotic balance to account for the lack of fumarate.

For *G. sulfurreducens*, kanamycin (200 µg/ml) was supplemented when needed unless otherwise stated. *Escherichia coli* K-12 strains were grown in Luria-Bertani medium (LB) supplemented with kanamycin (50 µg/ml) and 2,6-diaminopimelic acid (0.3 mM) when culturing *E. coli* strain BW29427 (this strain is also commonly referenced as WM3064).

**Electrode bioreactor setup.** Electrode bioreactors were assembled as previously described (18), with minor modifications. In all cases, 1500 grit wet/dry sandpaper (*Ali Industries, Inc.*, Fairborn, OH) was used to polish the 3-cm2 graphite working electrode. To remove residual graphite dust, polished electrodes were sonicated in ddH₂O with three water exchanges. Reactors were assembled using platinum wire counter electrodes and Calomel reference electrodes calibrated before each experiment against a master reference electrode. Assembled reactors were cleaned using alternating 1 N NaOH and 1 N HCl washes, with a final wash using ddH₂O to ensure a neutral pH. Reactors were then sterilized at 121°C for 20 min prior to use.

**Growth-independent Fe(III) citrate reduction assay.** Fe(III) citrate reduction was assayed in 10-ml anaerobic tubes or 96-well plates in real time with a buffered solution of Fe(III) citrate with FerroZine containing 7180 nmol of Fe(II)SO₄ as standards. In the real-time assay, before performing a FerroZine (2 g/liter in 50 mM HEPES) assay using 10-ml anaerobic tubes or 96-well plates. The assay was initiated by the addition of a 10:1 volume of the solution to a fully grown fumarate culture and incubated inside an Omni-Lab (Vacuum Atmospheres Company; 100% N₂ atmosphere) monitoring the absorbance every 1 min at 625 nm in a SpectraMax M2 ( Molecular Devices) over a 2-h period. Total cellular protein were determined by a bicinchoninic acid (Pierce) protein assay, and Fe(II)SO₄ standards in the real-time assay were digested with Ascl and NdeI and ligated into the same sites in pRV MCS-2. The *acpP* promoter replaced the vanR gene and vanA promoter in pRV MCS-2 to generate pRK2-Geo1. The *acpP* promoter sequence cloned in pRK2-Geo1 includes the *acpP* RBS.

**pRK2-Geo2 and pRK2-Geo2-lacZa.** A fragment of the *bla* gene was excised from pRVMCS-2 using BamHI and SacI, blunt-end filled, and religated to generate pRK2m1. The *nptI* gene was amplified with the primers nptI1 and nptI2 using pRVMCS-2 as a template and ligated into BbsI-digested and blunted pRK2m1 to generate pRK2m2. The *nptI* gene in pRK2m2 is in the reverse orientation as pRVMCS-2. Primer oriV-rmb-oriT (gBlock) containing an oriV and oriT sequence with the E. coli rmb transcription termination sequence was digested with Ascl and MscI and ligated into the same sites in pRK2m2 to generate pRK2m3. The *acpP* (GSU1604) promoter region of pRK2-Geo1 was digested with NotI and NheI and ligated into the same sites in pRK2m3 to generate pRK2-Geo2. The fragment containing the lacZa in pSRK-Km was excised from pSRK-Km and ligated into the same sites in pRK2-Geo2 to generate pRK2-Geo2-lacZa.

**pRK2-Geo2i and pRK2-Geo2i-lacZa.** Primer PGUS0800-vanR-PacP-vana (gBlock) containing the promoter regions of GSU0800 and a modified GSU1604 promoter region with VanR binding sites was digested with NcoI and NdeI and ligated into the same sites in pRVMCS-2 to generate pGV MCS-2. The modified promoter region in pGV MCS-2 was digested with NotI and NheI and ligated into the same sites in pRK2m3 to generate pRK2-Geo2i. The fragment containing the lacZa in pSRK-Km was excised from pSRK-Km and ligated into the same site in pRK2-Geo2 to generate pRK2-Geo2i-lacZa.

**pRK2-Geo5.** Primer taclac (gBlock) containing the taclac promoter region was digested with Ascl and NdeI and ligated into the same sites in pRK2-Geo2 to generate pRK2-Geo5. The taclac promoter sequence with the RBS is identical to that in pCD341 (19).

**Plasmid construction.** The primers and oligonucleotides used in the present study were ordered from IDT and are listed in Table 2. DNA was amplified using Phusion HSII polymerase (Thermo), digested with Fast-Digest restriction enzymes (Thermo), blunted with Fast DNA Repair kit (Thermo), and ligated with Fast-Link DNA ligase (Epizentrum). All plasmids were extracted from cells using PureYield plasmid miniprep (Promega), and sequences were verified with Sanger sequencing in the University of Minnesota Genomics Center (UMGC).

**pRK2-Geo1.** The *acpP* (GSU1604) promoter region was generated by annealing and extending the primers *acpP*1 and *acpP*2 in a PCR. The product was digested with Ascl and NdeI and ligated into the same sites in pRV MCS-2. The *acpP* promoter replaced the *vanR* gene and *vana* promoter in pRV MCS-2 to generate pRK2-Geo1. The *acpP* promoter sequence cloned in pRK2-Geo1 includes the *acpP* RBS.
of the Russian Academy of Sciences [http://bioinf.spbau.ru/spades]) and a reference *G. sulfurreducens* genome as a trusted contig. MUMmer (version 3.23 [http://mummer.sourceforge.net]) was used to isolate the plasmid sequences remaining in the unmatched SPAdes scaffolds. Plasmid circularity was confirmed via dot plot, and self-overlapping ends were manually trimmed. Sequences were reconfirmed by mapping reads to the assembled contigs using breseq (version 0.24rc6; Barrick Lab/University of Texas at Austin [http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing]) (20).

To accurately design primers for mutants and verify the outcome of deletion experiments, a complete genome of our laboratory strain was assembled. Genomic DNA (5 µg) was used to generate a 20-kb insert library for PacBio long-read sequencing, and the library was size selected to 7 kb with the BluePippin electrophoresis system (Sage Science). De novo assembly was performed with HGAP v3 (21) in SMRT Analysis v2.2 on filtered subreads >12 kb (minimum quality 0.8) to provide 100× coverage, polished using the entire read set (~190×) to >99.999% consensus concordance (QV 50) with three successive passes through Quiver (21). A total of 123 remaining indels were removed by mapping 85× coverage of 2×250-bp Illumina reads using Pilon v1.10 (22).

ΔGSU0299 genomic DNA (0.3 µg) was submitted to UMGC for a 2×250-bp paired-end MiSeq, demultiplexed to 2 million reads and mapped against our *G. sulfurreducens* genome using breseq with default parameters.

**NCBI accession numbers.** Complete read files for our laboratory strain, the AGSU0299 strain, and the plasmids have been deposited in the NCBI Sequence Read Archive database under accession numbers SRX1101230, SRX1101232, and SRX1101235, respectively. Plasmid sequences for pRK2-Geo2, pRK2-Geo2i, and pRK2-Geo5 have been deposited in the NCBI GenBank database with accession numbers KT339318, KT339319, and KT339320, respectively.

**RESULTS**

Generating a markerless deletion of GSU0299 to enable motility. FrgM is a regulator of flagellar synthesis (6), but in the *G.

---

**TABLE 2 Primers used in this study**

| Function and primer | Sequence (5′-3′) |
|---------------------|-----------------|
| **Complementation vectors** | |
| acpP1 | ACACGCGGCGGCTATTTTGAATTTAGGGCGTTTCGTGGTATGTAGCTAGAAACATTAC |
| acpP2 | CTGTCATATGGCTTGTTCACCTCCGTTTGGTTTGAGTCTTCGGGTAATGTTTCTAGCTAC |
| nptI1 | GAGACGTTGATCGGCACGTAAG |
| nptI2 | GGTGTTGCTGACTCTTGGTTTGAGTCTTCGGGTAATGTTTCTAGCTAC |
| oriV-rrnB-oriT | TAAATAGCCTCATAATAGGGCGCATGGACGAAATACTAGCGGCCACCCCTCATCTGTCAGTGAGGGCCAAGTTTTCC |
| **Deletion vectors** | |
| 0299-1F | CGCGGATCCAGCTCACGTCATGCCGTCAAG |
| 0299-1R | GCGGTTGAAGCGGTCGAGGAAGGTGTTGACCAGAAGGGGGATG |
| 0299-2F | CATCCCCCTTCTGGTCAACACCTTCCTCCGTGCCGGCCGCTACATGGCTCTGCCGACAAACAACGAAAGGAATTTTTCACGTTCGGCACCTCTCCTAAATGAATT |
| 0299-2R | CGACTAGTGCACAGTCTGCAATATTCGCTGATATC |
| imH-1F | CGCCGATCCGGACCTCAGCTAGCCGTCAG |
| imH-1R | GGAGGAGATGTATGACATTGCGCAAGCACTGACACGGCCTCGAG |
| imH-2F | CTGCAGGGCGTGTAGCTGCAATGCTCATACGCTTCCC |
| imH-2R | CGACTAGTGAAGATGCGACATCGTACATCTTCCC |
| imH-K1F | GCTAGATACGCAGTACGTCGATATC |
| imH-K1R | GGAGGAGATGTATGACATTGCGCAAGCACTGACACGGCCTCGAG |
| imH-K2F | CTGCAGGGCGTGTAGCTGCAATGCTCATACGCTTCCC |
| imH-K2R | GCTAGATACGCAGTACGTCGATATC |
| **Confirmation of gene deletion** | |
| 0299-up-F | TTACGTGCTCAGCAGCGCATTGCG |
| 0299-down-R | GTGTCATACGACAGGCCGAGAG |
| imH-up-F | CTGCCATACGCGCTGCTCAATCAACCGACAGTCGTCGATATC |
| imH-down-R | CCTCTACGTGCAGTACGTCGATATC |
| **imH vector** | |
| imH1 | ACGCCCATATGAGGTCGCGAAAACGGCAG |
| imH2 | CGCTCTGATTGTCGCTGACGGCGTGTCGAG |

---

of the Russian Academy of Sciences [http://bioinf.spbau.ru/spades]) and a reference *G. sulfurreducens* genome as a trusted contig, MUMmer (version 3.23 [http://mummer.sourceforge.net]) was used to isolate the plasmid sequences remaining in the uniliated SPAdes scaffolds. Plasmid circularity was confirmed via dot plot, and self-overlapping ends were manually trimmed. Sequences were reconfirmed by mapping reads to the assembled contigs using breseq (version 0.24rc6; Barrick Lab/University of Texas at Austin [http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing]) (20).

To accurately design primers for mutants and verify the outcome of deletion experiments, a complete genome of our laboratory strain was assembled. Genomic DNA (5 µg) was used to generate a 20-kb insert library for PacBio long-read sequencing, and the library was size selected to >7 kb with the BluePippin electrophoresis system (Sage Science). De novo assembly was performed with HGAP v3 (21) in SMRT Analysis v2.2 on filtered subreads >12 kb (minimum quality 0.8) to provide 100× coverage, polished using the entire read set (~190×) to >99.999% consensus concordance (QV 50) with three successive passes through Quiver (21). A total of 123 remaining indels were removed by mapping 85× coverage of 2×250-bp Illumina reads using Pilon v1.10 (22).

ΔGSU0299 genomic DNA (0.3 µg) was submitted to UMGC for a 2×250-bp paired-end MiSeq, demultiplexed to 2 million reads and mapped against our *G. sulfurreducens* genome using breseq with default parameters.

**NCBI accession numbers.** Complete read files for our laboratory strain, the AGSU0299 strain, and the plasmids have been deposited in the NCBI Sequence Read Archive database under accession numbers SRX1101230, SRX1101232, and SRX1101235, respectively. Plasmid sequences for pRK2-Geo2, pRK2-Geo2i, and pRK2-Geo5 have been deposited in the NCBI GenBank database with accession numbers KT339318, KT339319, and KT339320, respectively.

**RESULTS**

Generating a markerless deletion of GSU0299 to enable motility. FrgM is a regulator of flagellar synthesis (6), but in the *G.
**Development of stable replicating expression vectors for** *G. sulfurreducens*. Two broad-host-range backbones are routinely used for complementation in *G. sulfurreducens*. We compared the effects of pSRK (pBBR1 origin) and pRVMCS-2 (RK2 origin, IncP) on *G. sulfurreducens* growth in the presence of kanamycin (24, 25). Cells carrying the pBBR1 plasmid had a longer doubling time (∼33%) and demonstrated measurable lag compared to WT when fumarate was the terminal electron acceptor (Fig. 2A). The RK2 plasmid only slowed doubling times by ∼15% in the presence of kanamycin and showed no lag. The copy number was higher for pBBR1, at ∼50 copies per cell versus ∼5 copies per cell for RK2. This increased burden may contribute to the known instability of pBBR plasmids in *G. sulfurreducens* cells (9). Eighty-five percent of *G. sulfurreducens* cells retained the RK2 vector after over 15 generations in liquid acetate-fumarate medium in the absence of kanamycin selection by plating dilutions after liquid growth on plates containing kanamycin, whereas the pBBR1 vector was rapidly cured, similar to previous observations (9). Therefore, we selected RK2-based plasmids as the basis for a new expression system.

To generate constitutive expression vectors, we first replaced the *vanA* promoter, *vanR* and *bla* genes in the *Caulobacter crescent-

---

**FIG 1** Markerless deletion of GSU0299 from the *G. sulfurreducens* genome. (A) Flanking sequences of the GSU0299 transposon in the *sacB* vector pK18mobsacB integrate in this example upstream of GSU0299 in the presence of kanamycin. Cells plated on sucrose select for a second recombination event that can either generate the WT or the deletion allele. (B) Primers p1 to p4 depicted in panel A screen for deletion of GSU0299. (C) Full genome sequencing found 150× coverage of reads spanning the deletion junction in *fgrM*, verifying the transposon was removed. No reads spanned the WT junction, and no other mutations were found in the ΔGSU0299 genome. (D) The resulting ΔGSU0299, *fgrM* 

---

$sulfurreducens$ genome, the bona fide *fgrM* gene is interrupted by a transposase (GSU0299), inactivating motility. We selected GSU0299 as a target for a SacB/sucrose counterselection strategy as a proof-of-concept, as this required a precise scarless in-frame deletion for gain of function.

*E. coli* conjugation donor strains are able to transfer plasmids containing mobilization genes (*mob*) and an origin of transfer (*oriT*) into *G. sulfurreducens* (5). In preliminary screening, the S17-1 *E. coli* donor strain was more efficient in transferring *mob* vectors into *G. sulfurreducens* compared to the previously reported *E. coli* donor strain BW29427. The *sacB* and *kanR* encoding vectors pSMV3 and pK18mobsacB, which are unable to replicate in *G. sulfurreducens*, were engineered to contain homologous regions flanking GSU0299. These plasmids integrated into either flanking region of GSU0299 when transconjugants were plated on medium containing kanamycin. Strains in which removal of GSU0299 occurred via a second recombination event were selected by growing transconjugants on 10% sucrose medium (Fig. 1A). The activity of SacB in the presence of sucrose was inhibitory to *G. sulfurreducens* (23), and sucrose-resistant colonies were screened for kanamycin sensitivity, indicating loss of the plasmid. Approximately 20% of isolates were still resistant to kanamycin after sucrose selection, likely due to inactivation of *sacB*, while PCR screening and sequencing revealed 50% of the kanamycin-sensitive isolates had the desired mutation (Fig. 1B).

The resulting ΔGSU0299 strain spread from sites of inoculation in motility medium containing 0.4% agar (Fig. 1D). Full genome resequencing confirmed the ΔGSU0299 deletion was in-frame and was the only mutation compared to the parent strain (Fig. 1C). Ten times more *G. sulfurreducens* transconjugants were isolated using the higher-copy-number *sacB* plasmid pK18mobsacB compared to pSMV3. A similar approach was then used with pK18mobsacB to delete *imcH*, an inner membrane cytochrome required for reduction of high redox potential electron acceptors such as Fe(III) citrate, Mn(IV) oxides, and electrodes poised at potentials > −0.1 V versus SHE (7). The subsequent Δ*imcH* strain was used to study the different vectors and promoters useful in complementation and induced expression.
plasmid pRVMCS-2 with the G. sulfurreducens acpP (GSU1064) promoter or the E. coli based tacIac promoter to generate pRK2-Geo2 and pRK2-Geo5, respectively (Fig. 2C). Both acpP transcripts and the AcpP protein are highly abundant across multiple conditions, and its transcription is only regulated by the housekeeping sigma factor RpoD (26, 27). The native acpP promoter was modified to include palindromic VanR binding sites, and expression of vanR was driven by another constitutive RpoD-dependent promoter (GSU0800), creating the vanillate-inducible vector pRK2-Geo2i (Fig. 2D) (25).

In preliminary work, cloning imcH under the control of the C. crescentus vanA promoter in pRVMCS-2 did not complement growth of the ΔimcH strain in Fe(III) citrate in the presence or absence of vanillate, suggesting that Geobacter did not recognize the Caulobacter vanA or vanR promoters. When the constitutive G. sulfurreducens acpP (GSU1604) promoter was used to express imcH in the pRK2-Geo2 backbone (pLMCH15), this construct fully complemented growth of the ΔimcH strain (Fig. 3A). When imcH was cloned into the inducible pRK2-Geo2i vector (pLMCH16), reduction of Fe(III) by the ΔimcH strain was triggered even when vanillate was added 24 h after inoculation (Fig. 3A). To further confirm the pRK2-Geo2i-inducible vector could be used in the study of insoluble electron acceptors, we demonstrated that Mn(IV) reduction only occurred in ΔimcH/pLMCH16 cultures when vanillate was added (Fig. 3B) (7). In addition, both the constitutive acpP and the modified vanillate-inducible promoters in pRK2-Geo2 and pRK2-Geo2i were recognized by E. coli. When the lacZα fragment was cloned downstream of acpP, blue colonies were produced on LB X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) plates. In the pRK2-Geo2i-lacZα construct, E. coli colonies turned blue only in the presence of vanillate.

While studying growth of kanamycin cassette insertion mutants on electrodes, we noticed a correlation between low inoculum levels and higher growth rates and hypothesized that this was due to reduced carryover of antibiotics. To test this effect further, WT cells containing pRK2-Geo2i were precultured in fumarate medium, and after dilution into a poised electrode bioreactor the concentration of antibiotic carried over was 5 μg/ml. Additional kanamycin was then added to bioreactors to concentrations typically used for mutant and plasmid selection (200 μg/ml), as well as a concentration representing only 25% of this level. Even lower concentrations of antibiotic significantly slowed growth and decreased the final current density of the biofilms (Fig. 2B). These experiments confirmed that, even with an appropriate resistance gene present, sublethal levels of kanamycin alter the outcome of experiments. For subsequent experiments, all preculturing and inoculation was done to achieve <5 μg of kanamycin/ml in electrode reactors, since these plasmids are maintained for multiple generations without antibiotic selection.

Compared to planktonic growth with Fe(III) citrate, induction
in biofilms may respond differently. To determine whether the vanillate system could be used to restore wild-type rates of electron transfer to electrodes, \textit{H.9004} imcH cells carrying the inducible plasmid pImcH16 were inoculated into bioreactors poised at 0.24 V versus SHE. The addition of vanillate after inoculation immediately triggered growth. However, a higher concentration than what was used for Fe(III) citrate was needed to reach WT growth rates and biofilm current densities (Fig. 3C).

High-throughput Fe(III) citrate reduction assay. A spectrophotometric 96-well plate assay was developed to quickly compare rates of Fe(III) citrate reduction and screen the array of plasmids, promoters, and mutants created in this study (28). This real-time assay measures Fe(III) reduction independent of growth due to a lack of phosphate, nitrogen, mineral, and carbon dioxide sources in the reaction. The assay is initiated by the addition of cells directly in a Ferrozine-containing, Fe(III) citrate buffer. Previously, our growth independent Fe(III) citrate reduction assay was performed in 10-ml Balch tubes for 4 h, requiring samples to be taken by syringe per time point and analyzed via mixing with Ferrozine reagent. In a direct comparison of these methods, rates of Fe(III) citrate reduction per mg of protein by WT cells in the 96-well plate method were similar to rates measured using the longer 10-ml Balch tube assay (Fig. 4).

**FIG 3** Vanillate induction under Fe(III) citrate, Mn(IV) oxide, and poised electrode growth conditions. (A) Growth using Fe(III) citrate as the terminal electron acceptor in WT carrying the empty vector pRK2-Geo2i (●) and \textit{ΔimcH} strain carrying plmcH16 (imcH\textsuperscript{+} in pRK2-Geo2i). \textit{ΔimcH}/plmcH16 cells were induced with 50 μM vanillate at 24 h (▲) or left uninduced (○). (Results are indicated as ± the SD; \textit{n} = 3 for each condition). (B) Mn(IV) oxide reduction by WT \textit{G. sulfurreducens} carrying pRK2-Geo2i (●) compared to \textit{ΔimcH} carrying plmcH16 with (■) and without (○) induction by 50 μM vanillate. (Results are indicated as ± the SD; \textit{n} = 3 for each condition). (C) Current density on electrodes poised at 0.24 V versus SHE at 80 h, comparing WT carrying empty pRK2-Geo2i to \textit{ΔimcH} carrying pRK2-Geo2i, and \textit{ΔimcH} carrying the vanillate-inducible plmcH16 with 50 and 100 μM vanillate added at the time of inoculation. (Results are indicated as ± the SD, each point = one independent reactor; \textit{n} = 27 reactors in total).
To demonstrate the utility of the real-time assay, complementation of the $\Delta imcH$ strain by two different complementation vectors, pLmH15 (pRK2-Geo2), and pLmH17 (pRK2-Geo5), along with the inducible pLmH16 (pRK2-Geo2i) was compared. Both pLmH15 and pLmH17 restored near WT Fe(III) citrate reduction rates, and increasing rates of Fe(III) reduction were achieved by increasing vanillate concentrations from 0 to 10 $\mu$M (Fig. 4). The concentrations required to elicit full induction in the Geobacter VanR-inducible system were similar to those previously reported in Caulobacter and Myxococcus, with the response range in the low-micromolar levels (25, 29). A background rate of Fe(III) reduction in the absence of vanillate was detectable in the non-growth assay, suggesting a low level of $imcH$ expression from pLmH16 or background activity due to other inner membrane cytochromes. Since growth on Fe(III) citrate was not detected in the same strain in the absence of vanillate (Fig. 3A), this demonstrated the increased sensitivity of the nongrowth real-time Fe(III) citrate reduction assay in detecting residual electron transfer activity in mutants.

**DISCUSSION**

Precise removal and insertion of DNA into the *G. sulfurreducens* chromosome enabled by the SacB/sucrose counter selection strategy allows for construction of multigene deletion and insertion mutants without the effects of polarity or undesired changes in the chromosome. While weeks of anaerobic manipulation are required to generate a mutation using the two-step method, the resulting strain is not burdened by expression of antibiotic resistance cassettes, increasing the availability of markers for maintenance of expression plasmids. Researchers should take caution in the use of antibiotics, since we show the aminoglycoside kanamycin is inhibitory even when cells are expressing a resistance gene. Gentamicin, another aminoglycoside, showed similar inhibitory effects on cell growth even when respiring to a soluble electron acceptor in our preliminary studies, and therefore was not considered as we developed plasmids for this work. This underscores the need for proper empty-vector controls conducted under similar conditions in genetic studies.

Ectopic expression in *G. sulfurreducens* previously relied on broad-host-range plasmids of unknown copy number from promoters with unknown strengths, which could partly explain the incomplete complementation of mutations in Geobacter (5, 7, 13). By using a more stable, low-copy-number plasmid and modifying a constitutively expressed promoter (acpP), we have the ability to control gene expression by the addition of a small, nontoxic, membrane permeable molecule. Since we developed these vectors using a crucial respiratory cytochrome as the benchmark for full complementation, analyses of other physiologically relevant genes involved in metal respiration should be possible. For more complex genetic circuitry or multigene expression analysis, future work will need to engineer additional inducible expression systems with other membrane permeable compounds such as benzoate or short-chain fatty acids.

To accelerate the development of expression vectors and analyze the complex electron transfer pathway of Geobacter, a sensitive kinetic assay is necessary. A real-time Fe(III) citrate assay proved to be faster and use less reagent than the discontinuous assay. This nongrowth assay appears to detect residual rates of electron transfer in $\Delta imcH$ mutants that were too slow to support growth and that were not easily detectable in the traditional assay.

Part of this increased sensitivity could come from the presence of the Fe(II)-trapping reagents poising the Fe(III)/Fe(II) ratio consistently high, keeping electron transfer favorable. This sensitivity and consistency will be useful in the search for secondary or overlapping electron transfer mechanisms.

The genetic tools presented here will aid future genetic manipulation of *G. sulfurreducens*, and minimize confounding factors such as antibiotic inhibition or stability of vectors. This system offers a much needed ability to directly manipulate gene expression levels in Geobacter and provides an example of how Geobacter can be engineered to produce electrical current upon sensing an external signal (30). This combination of genetic precision and transcriptional control is a crucial part of any biosensor or biodevice based on extracellular electron transfer.

**ACKNOWLEDGMENTS**

We thank P. Mera in the L. Shapiro laboratory, M. Spero in the T. Donohue laboratory, and J. Will in the J. Escalante-Semerena laboratory for providing strains and plasmids used in this work.

This study was supported by grant N000141210308 from the Office of Naval Research and grant DE-SC0006868 from the Department of Energy (Biological and Environmental Research).

**REFERENCES**

1. Brophy JAN, Voigt CA. 2014. Principles of genetic circuit design. Nat Methods 11:508–520. http://dx.doi.org/10.1038/nmeth.2926.

2. Leigh JA, Albers S-V, Atomi H, Allers T. 2011. Model organisms for genetics in the domain Archaea: methanogens, halophiles, Thermococcales and Sulfolobales. EMS Microbiol Rev 35:577–608. http://dx.doi.org/10.1111/j.1574-6976.2011.00265.x.

3. Woodson JD, Escalante-Semerena JC. 2004. CbiZ, an amidohydrolase enzyme required for salvaging the coenzyme $B_2$, precursor cobinamide in archaea. Proc Natl Acad Sci U S A 101:3591–3596. http://dx.doi.org/10.1073/pnas.0305939101.

4. McMahon MD, Guan C, Handelsman J, Thomas MG. 2012. Metagenomic analysis of *Streptomyces lividans* reveals host-dependent functional expression. Appl Environ Microbiol 78:3622–3629. http://dx.doi.org/10.1128/AEM.00444-12.

5. Rollefson JB, Stephen CS, Tien M, Bond DR. 2011. Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in Geobacter sulfurreducens. J Bacteriol 193:1023–1033. http://dx.doi.org/10.1128/JB.01092-10.

6. Ueki T, Leang C, Inoue K, Lovley DR. 2012. Identification of multicomponent histidine-aspartate phosphorelay system controlling flagellar and motility gene expression in *Geobacter* species. J Biol Chem 287:10958–10966. http://dx.doi.org/10.1074/jbc.M111.345041.

7. Levar CE, Chan CH, Mehta-Kolte MG, Bond DR. 2014. An inner membrane cytochrome required only for reduction of high redox potential extracellular electron acceptors. mBio 5:e02034. http://dx.doi.org/10.1128/mBio.02034-14.

8. Mehta T, Coppi MV, Childers SE, Lovley DR. 2005. Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in Geobacter sulfurreducens. Appl Environ Microbiol 71:8634–8641. http://dx.doi.org/10.1128/AEM.71.12.8634-8641.2005.

9. Coppi MV, Leang C, Sandler SJ, Lovley DR. 2001. Development of a genetic system for Geobacter sulfurreducens. Appl Environ Microbiol 67:3180–3187. http://dx.doi.org/10.1128/AEM.67.11.3180-3187.2001.

10. Voorderckers JW, Kim B-C, Izallalen M, Lovley DR. 2010. Role of Geobacter sulfurreducens outer surface c-type cytochromes in reduction of soil humic acid and anthraquinone-2,6-disulfonate. Appl Environ Microbiol 76:2371–2375. http://dx.doi.org/10.1128/AEM.02250-09.

11. Rollefson JB, Levar CE, Bond DR. 2009. Identification of genes involved in biofilm formation and respiration via mini-Himar transposon tagging of Geobacter sulfurreducens. J Bacteriol 191:4207–4217. http://dx.doi.org/10.1128/JB.00057-09.

12. Mahadevan R, Bond DR, Butler JE, Esteve-Nuñez A, Coppi MV, Palsson BO, Shilling CH, Lovley DR. 2006. Characterization of metabolism in the Fe(III)-reducing organism Geobacter sulfurreducens by con-
13. Leang C, Coppi MV, Lovley DR. 2003. OmcB, a c-type polyheme cytochrome, involved in Fe(III) reduction in Geobacter sulfurreducens. J Bacteriol 185:2096–2103. http://dx.doi.org/10.1128/JB.185.7.2096-2103.2003.

14. Tremblay PL, Aklujkar M, Leang C, Nevin KP, Lovley D. 2012. A genetic system for Geobacter metallireducens: role of the flagellin and pilin in the reduction of Fe(III) oxide. Environ Microbiol Rep 4:82–88. http://dx.doi.org/10.1111/j.1758-2229.2011.00305.x.

15. Summers ZM, Ueki T, Ismail W, Haveman SA, Lovley DR. 2012. Laboratory evolution of Geobacter sulfurreducens for enhanced growth on lactate via a single-base-pair substitution in a transcriptional regulator. ISME J 6:975–983. http://dx.doi.org/10.1038/ismej.2011.166.

16. Marx CJ, Lidstrom ME. 2002. Broad-host-range cre-lox system for anti-biotic marker recycling in Gram-negative bacteria. Biotechniques 33: 1062–1067.

17. Lovley DR, Phillips EJ. 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol 51: 683–689.

18. Marsili E, Rollefson JB, Baron DB, Hozalski RM, Bond DR. 2008. Microbial biofilm voltammetry: direct electrochemical characterization of catalytic electrode-attached biofilms. Appl Environ Microbiol 74:7329–7337. http://dx.doi.org/10.1128/AEM.00177-08.

19. Dehio M, Knorre A, Lanz C, Dehio C. 1998. Construction of versatile high-level expression vectors for Bartonella henselae and the use of green fluorescent protein as a new expression marker. Gene 215:223–229. http://dx.doi.org/10.1016/S0378-1119(98)00319-9.

20. Barrick JE, Colburn G, Deatherage DE, Traverse CG, Strand MD, Borges JJ, Knoester DB, Reb A, Meyer AG. 2014. Identifying structural variation in haploid microbial genomes from short-read resequencing data using bresq. BMC Genomics 15:1039. http://dx.doi.org/10.1186/1471-2164-15-1039.

21. Chin C-S, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, Clum A, Copeland A, Huddleston J, Eichler EE, Turner SW, Korlach J. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods 10:563–569. http://dx.doi.org/10.1038/nmeth.2474.

22. Walker BJ, Abeel T, Shea T, Priest M, Abouelil A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK, Earl AM. 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 9:e112963. http://dx.doi.org/10.1371/journal.pone.0112963.

23. Gay P, Le Coq D, Steinmetz M, Berkelman T, Kado CI. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J Bacteriol 164:918–921.

24. Khan SR, Gaines J, Roop RM, Farrand SK. 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraK and TraM expression on Ti plasmid quorum sensing. Appl Environ Microbiol 74:5053–5062. http://dx.doi.org/10.1128/AEM.01098-08.

25. Thanhchilier M, Iniesta AA, Shapiro L. 2007. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in Caulobacter crescentus. Nucleic Acids Res 35:e137. http://dx.doi.org/10.1093/nar/gkm818.

26. Ding Y-HR, Hisson KK, Giometti CS, Stanley A, Esteve-Núñez A, Khare T, Tollaksen SL, Zhu W, Adkins JN, Lipton MS, Smith RD, Mester T, Lovley DR. 2006. The proteome of dissimilatory metal-reducing microorganism Geobacter sulfurreducens under various growth conditions. Biochim Biophys Acta 1764:1198–1206. http://dx.doi.org/10.1016/j.bbapap.2006.04.017.

27. Qiu Y, Nagarajan H, Embree M, Shieu W, Abate E, Juárez K, Cho B-K, Elkins JG, Nevin KP, Barrett CL, Lovley DR, Palsson BO, Zengler K. 2013. Characterizing the interplay between multiple levels of organization within bacterial sigma factor regulatory networks. Nat Commun 4:1755. http://dx.doi.org/10.1038/ncomms2743.

28. Childers SE, Lovley DR. 2001. Differences in Fe(III) reduction in the hyperthermophilic archaean, Pyrobaculum islandicum, versus mesophilic Fe(III)-reducing bacteria. FEMS Microbiol Lett 195:253–258. http://dx.doi.org/10.1111/j.1574-6968.2001.tb10529.x.

29. Iniesta AA, García-Heras F, Abellón-Ruiz J, Gallego-Garcia A, Elias-Arnanz M. 2012. Two systems for conditional gene expression in Myxococcus xanthus inducible by isopropyl-β-D-thiogalactopyranoside or vanillate. J Bacteriol 194:5875–5885. http://dx.doi.org/10.1128/JB.01110-12.

30. Goltsch F, Bücking C, Gescher J. 2013. Proof of principle for an engineered microbial biosensor based on Shewanella oneidensis outer membrane protein complexes. Biosens Bioelectron 47:285–291. http://dx.doi.org/10.1016/j.bios.2013.03.010.

31. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat Biotechnol 1:784–791. http://dx.doi.org/10.1038/nbt1183-784.

32. Saltkov CW, Cifuentes A, Venkatesswaran K, Newman DK. 2003. The ars detoxification system is advantageous but not required for As(V) respiration by the genetically tractable Shewanella species strain ANA-3. Appl Environ Microbiol 69:2800–2809. http://dx.doi.org/10.1128/AEM.69.5.2800–2809.2003.