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Published in:
Environmental Microbiology Reports

Link to article, DOI:
10.1111/j.1758-2229.2011.00305.x

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

environmental microbiology reports

Environmental Microbiology Reports (2012) **4**(1), 82–88 doi:10.1111/j.1758-2229.2011.00305.x

A genetic system for *Geobacter metallireducens*: role of the flagellin and pilin in the reduction of Fe(III) oxide

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Summary

***Geobacter metallireducens* is an important model organism for many novel aspects of extracellular electron exchange and the anaerobic degradation of aromatic compounds, but studies of its physiology have been limited by a lack of techniques for gene deletion and replacement. Therefore, a genetic system was developed for *G. metallireducens* by making a number of modifications in the previously described approach for homologous recombination in *Geobacter sulfurreducens*. Critical modifications included, among others, a 3.5-fold increased in the quantity of electrotransformed linear DNA and the harvesting of cells at early-log. The Cre-*lox* recombination system was used to remove an antibiotic resistance cassette from the *G. metallireducens* chromosome permitting the generation of multiple mutations in the same strain. Deletion of the gene *fliC*, which encodes the flagellin protein, resulted in a strain that did not produce flagella, was non-motile, and was defective for the reduction of insoluble Fe(III). Deletion of *pilA*, which encodes the structural protein of the type IV pili, inhibited the production of lateral pili as well as Fe(III) oxide reduction and electron transfer to an electrode. These results demonstrate the importance of flagella and pili in the reduction of insoluble Fe(III) by *G. metallireducens* and provide methods for additional genetic-based approaches for the study of *G. metallireducens*.**

Introduction

Elucidation of the physiology of *Geobacter metallireducens* strain GS-15 is of interest because this organism has provided the first example of a number of previously undescribed forms of microbial metabolism. It was the first microorganism found to conserve energy to support growth from the oxidation of organic compounds coupled to the reduction of Fe(III) or Mn(IV) oxides (Lovley *et al*., 1987; Lovley and Phillips, 1988); the first pure culture found to oxidize aromatic hydrocarbons anaerobically (Lovley *et al*., 1989; Lovley and Lonergan, 1990); the first microorganism found to reduce U(VI) (Lovley *et al*., 1991) or humic substances (Lovley *et al*., 1996) as an electron acceptor; and one of the first microorganisms found to oxidize organic compounds completely to carbon dioxide with electron transfer to an electrode (Bond *et al*., 2002). It was the first *Geobacter* species isolated in pure culture (Lovley *et al*., 1993) and served as an early model for the metabolism of the *Geobacter* species that are important components of anaerobic soils and sediments.

However, *Geobacter sulfurreducens* became the *Geobacter* species of choice for most studies when a genetic system for *G. sulfurreducens* was developed (Coppi *et al*., 2001). For example, the functions of several *c*-type cytochromes and other proteins involved in extracellular electron transfer were characterized in *G. sulfurreducens* by evaluating the phenotype of gene deletions (Leang *et al*., 2003; Lloyd *et al*., 2003; Butler *et al*., 2004; Mehta *et al*., 2005; 2006; Holmes *et al*., 2006; Nevin *et al*., 2009; Voordeckers *et al*., 2010). Other biological processes were also studied with a functional genetic approach such as interspecies direct electron transfer (Summers *et al*., 2010); anchoring of *c*-type cytochromes in the extracellular matrix (Rollefson *et al*., 2011); acetate uptake (Risso *et al*., 2008b) and oxidation (Coppi *et al*., 2007); hydrogen oxidation (Coppi *et al*., 2004); fumarate reduction (Butler *et al*., 2006); isoleucine biosynthesis (Risso *et al*., 2008a); and various mechanisms of regulation (Nunez *et al*., 2004; Kim *et al*., 2005; 2006; DiDonato *et al*., 2006; Ueki and Lovley, 2007; 2010a,b; Juarez *et al*., 2009; Leang *et al*., 2009).

Geobacter sulfurreducens lacks many interesting physiological features of *G. metallireducens*. Most notably, *G. sulfurreducens* does not reduce Fe(III) oxide as effectively as *G. metallireducens* and lacks the ability to metabolize aromatic compounds (Caccavo *et al*., 1994; Aklujkar *et al*., 2009). Furthermore, *G. sulfurreducens* is

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non-motile (Caccavo *et al*., 1994), eliminating the possibility of examining the novel chemotaxis observed in *G. metallireducens* (Childers *et al*., 2002).

A previous study demonstrated that it was possible to express a heterologous gene from a plasmid introduced into *G. metallireducens* (Butler *et al*., 2006), but until now no system for gene deletion had been developed. Here we report the development of a genetic system for *G. metallireducens* and the use of this system to evaluate the role of flagella and pili in Fe(III) oxide reduction.

Results and discussion

Genetic system for G. metallireducens

Attempts to make gene deletions in *G. metallireducens* with the same protocol (Coppi *et al*., 2001; Lloyd *et al*., 2003; Nevin *et al*., 2009) that is effective with *G. sulfurreducens* failed repeatedly. Evaluation of each step of the *G. sulfurreducens* protocol resulted in a modified protocol (see Appendix S1, Fig. S3, Tables S1 and S2) that was successful in *G. metallireducens*. Important modifications included: increasing the amount of linear DNA used for electroporation by 3.5-fold; harvesting cells at early-log instead of mid-log; lower concentration of sucrose in the electroporation buffer; and amending acetate-Fe(III) citrate medium with yeast extract and ferrous ammonium sulfate for recovery of electrotransformed cells.

Application of the Cre-*lox* strategy (Marx and Lidstrom, 2002) to generate a markerless deletion in combination with the new protocol was successful in *G. metallireducens*, offering the possibility of generating strains with multiple mutations (see Appendix S1 and Fig. S3).

Importance of flagella in reduction of insoluble Fe(III)

The protocol was first evaluated in a study to understand the importance of flagella in Fe(III) oxide reduction. This is of interest because *G. metallireducens* reduces Fe(III) oxide 17 times faster (Tremblay *et al*., 2011) than *G. sulfurreducens*, which might be related to the specific expression of flagella by *G. metallireducens* during growth on Fe(III) oxide (Childers *et al*., 2002), whereas *G. sulfurreducens* is non-motile (Caccavo *et al*., 1994). It has been proposed that *Geobacter* species need flagellum-associated motility to hunt for Fe(III) oxides during growth in the subsurface (Childers *et al*., 2002; Esteve-Nunez *et al*., 2008; Lovley, 2008). The monocistronic gene *fliC*, which encodes the flagellin protein (Macnab, 2003; Tran *et al*., 2008) was replaced by a *fliC* mutant allele in which a spectinomycin resistance cassette replaced the coding sequence. PCR of genomic DNA confirmed that isolates of the mutant strain possessed the spectinomycin resistance cassette in the correct location and no longer possessed the coding sequence of *fliC* (Fig. S1).

The deletion of *fliC* prevented *G. metallireducens* from producing flagella during growth on Fe(III) oxide (Fig. 1A and B). Wild-type *G. metallireducens* grown on a soft agar plates in which Fe(III) citrate was provided as an electron

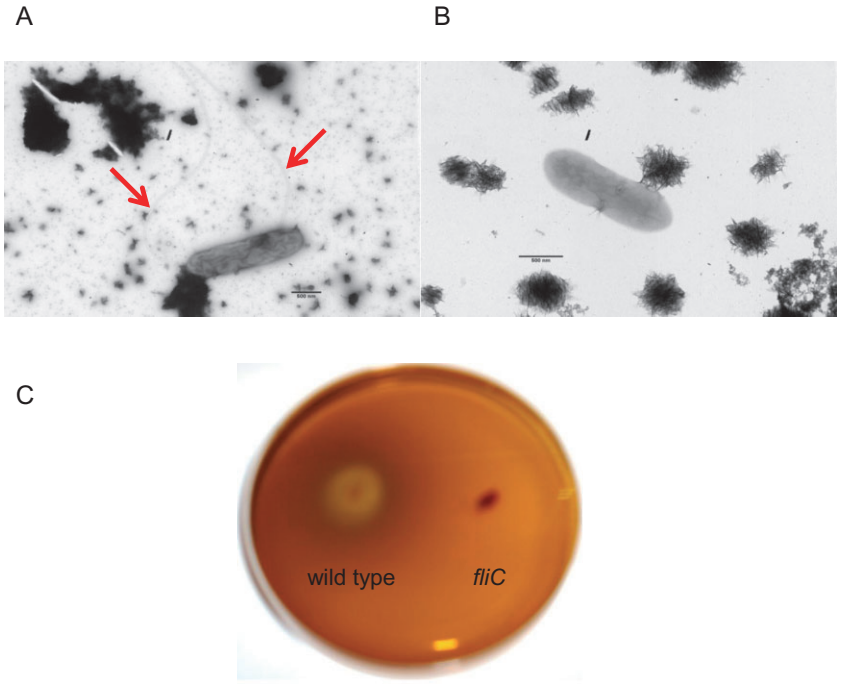

Fig. 1. Phenotype of the *G. metallireducens* flagellin (*fliC*) mutant during growth on Fe(III) oxide.
A. Transmission electron micrographs showing the presence of lateral flagella in the wild-type. Red arrows point towards the flagella.
B. Absence of flagella in the *fliC* mutant. Scale bars are 500 nm.
C. Growth and Fe(III) reduction after 2 weeks of incubation on soft agar plates with Fe(III) citrate as the electron acceptor. The larger clearing zone with the wild-type illustrates its motility through the agar.

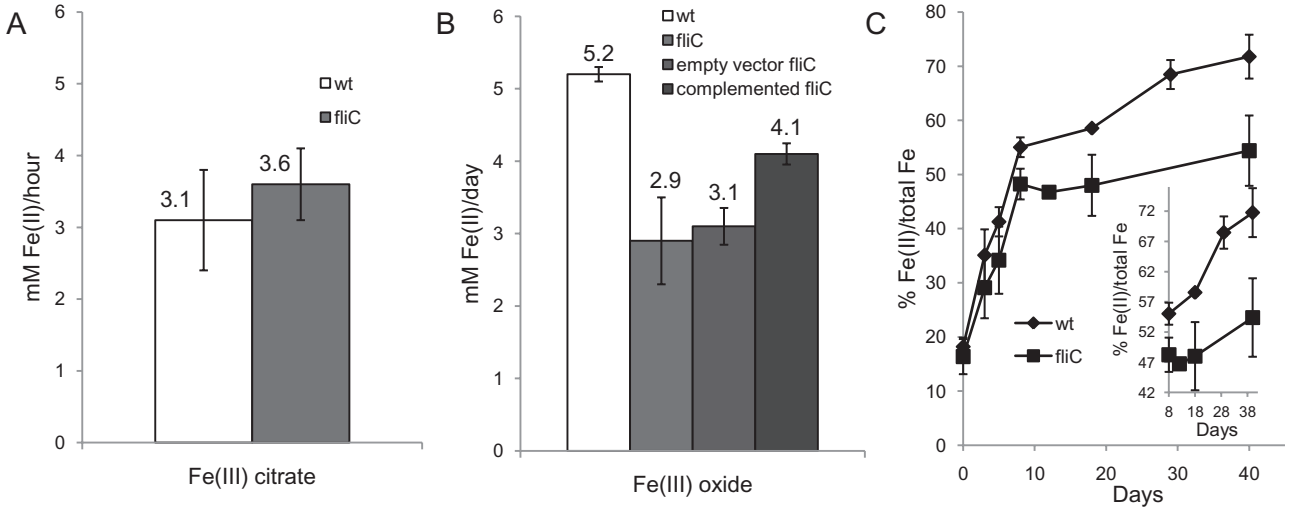

Fig. 2. Impact of *fliC* deletion on reduction of Fe(III). Fe(III) reduction rates when *G. metallireducens* wild-type and *fliC* mutant were grown with Fe(III) citrate (A). Fe(III) reduction rates when *G. metallireducens* wild-type, *fliC* mutant, *fliC* mutant with an empty vector and complemented *fliC* mutant were grown with synthetic Fe(III) oxide (B) as an electron acceptor. (C) Production of Fe(II) over time when *G. metallireducens* wild-type and *fliC* mutant were inoculated into sterile subsurface sediments The inset in (C) is a magnification of the data for days 8 to 40. Data are the mean of at least three independent experiments. Error bars represent the standard deviation of the mean.

acceptor migrated away from the original inoculation point, producing a zone of clearing where Fe(III) was reduced (Fig. 1C). In contrast, the strain with the defective *fliC* did not migrate (Fig. 1C), despite the fact that the mutant strain reduced Fe(III) citrate as well as wild-type (Fig. 2A). Deletion of *fliC* decreased the rate that poorly crystalline Fe(III) oxide synthesized in the laboratory (Lovley and Phillips, 1986) was reduced by nearly 45% compared with wild-type (Fig. 2B). Complementation of the *fliC* mutant with a plasmid expressing *fliC* from a constitutive *lac* promoter significantly increased the rate of Fe(III) oxide reduction (Fig. 2B).

Much of the Fe(III) in subsurface sediments is expected to be heterogeneously dispersed and thus to require more motility to access than synthetic Fe(III) oxide. In order to evaluate this, wild-type and *fliC*-deficient mutant cells were grown to mid-log in Fe(III) oxide medium and then inoculated (2%) into heated-sterilized subsurface sediments. Both strains initially reduced sediment Fe(III) at similar rates (Fig. 2C). However, after 8 days of incubation most of the readily reducible Fe(III) was depleted and rates of Fe(III) reduction slowed (Fig. 2C). During this second phase of sediment Fe(III) reduction the rate of Fe(III) reduction of wild-type cells (0.6% $\pm$ 0.1% of total Fe reduced to Fe(II) per day) was much faster than that of the *fliC*-deficient mutant (0.2% $\pm$ 0.2%). These results suggest that motility enhances the ability of *G. metallireducens* to access and reduce Fe(III) in sediments, especially as the availability of this electron acceptor becomes limited.

Role of pilA *in Fe(III) oxide reduction*

The potential importance of pili in Fe(III) reduction by *Geobacter* species was first noted in studies with *G. metallireducens* (Childers *et al*., 2002), but the role of pili in extracellular electron transfer has only been genetically evaluated in *G. sulfurreducens*, in which deletion of *pilA*, encoding the structural protein for the type IV pili inhibited reduction of Fe(III) oxide, but not reduction of soluble Fe(III) citrate (Reguera *et al*., 2005), and inhibited electron transfer to electrodes (Reguera *et al*., 2006; Nevin *et al*., 2009). Further evidence for the importance of pili in extracellular electron transfer was the finding that placing *G. sulfurreducens* under selective pressure for rapid Fe(III) oxide reduction (Tremblay *et al*., 2011) or electron transfer to electrodes (Yi *et al*., 2009) yielded strains with enhanced pilin production. The pili have organic metallic-like conductivity, which appears to account for their ability to facilitate electron transfer along their length (Malvankar *et al*., 2011).

In order to determine if pili play an important role in *G. metallireducens*, the *pilA* of *G. metallireducens* was mutated with the Cre-*lox* strategy (Marx and Lidstrom, 2002), which permitted removal of the spectinomycin resistance cassette after the gene was disrupted. PCR of genomic DNA confirmed that isolates of the mutant strain possessed the spectinomycin resistance cassette in the correct location and no longer possessed the coding sequence of *pilA*; upon introduction of the Cre recombinase expression plasmid, deletion of the spectinomycin

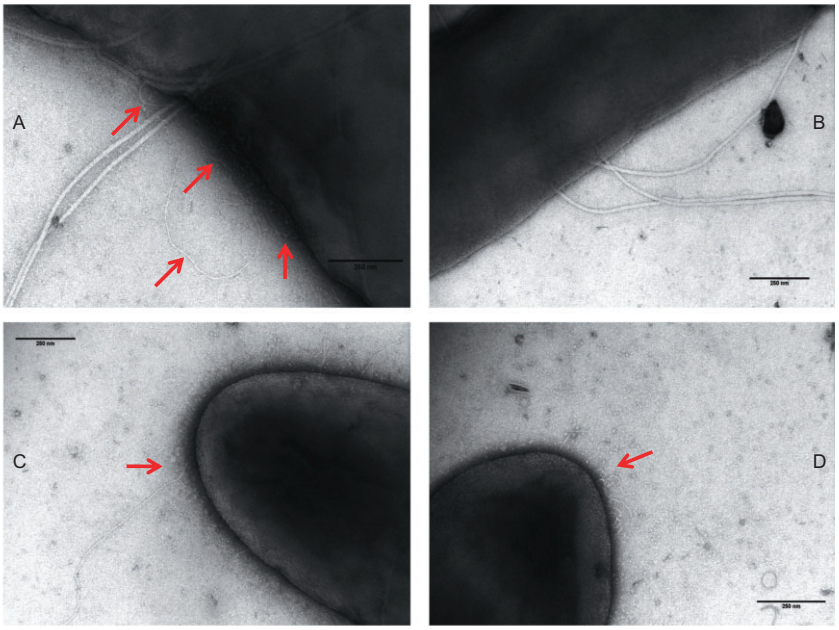

Fig. 3. Phenotype of the *G. metallireducens pilA* mutant. Transmission electron micrographs showing the presence of lateral pili in the wild-type (A) and the absence of lateral pili in a *G. metallireducens ΔpilA::loxP* strain (B). Pilus-like structures are found at the poles of wild-type (C) and *pilA* mutant cells (D). Red arrows indicate the position of pili. Scale bars are 250 nm. Both strains were grown with Fe(III) citrate as an electron acceptor at 25°C, a temperature favourable to pilin synthesis in *Geobacteraceae*.

resistance cassette was evident (Fig. S2). Deletion of *pilA* prevented expression of lateral pili (Fig. 3A and B). However, both the wild-type and the mutants have pili at their poles (Fig. 3C and D). The *G. sulfurreducens pilA* mutant also retains pilus-like filaments, which are thought to be implicated in cell attachment to surfaces (Klimes *et al*., 2010). Deletion of *G. metallireducens pilA* did not affect the rate of soluble Fe(III) citrate reduction (Fig. 4A), but the capacity for reduction of insoluble Fe(III) oxide was completely abolished (Fig. 4B). Complementation with a functional *pilA* gene expressed from a constitutive *lac* promoter restored the capacity for Fe(III) oxide reduction (Fig. 4B). The *pilA* mutant was also unable to transfer electrons to an electrode (Fig. 4C). These results demonstrate that, like *G. sulfurreducens* (Reguera *et al*., 2005; 2006; Nevin *et al*., 2009), *G. metallireducens* requires type IV pili for electron transfer to Fe(III) oxide or through anode biofilms.

Transcription of the *pilA* gene of *G. sulfurreducens* is initiated at two distinct sites, suggesting that translation may also be initiated at two sites, resulting in two isoforms of the PilA preprotein that are processed into an identical mature protein by removal of the signal peptide (Juarez *et al*., 2009). The PilA preprotein of *G. metallireducens* is 60 amino acids long, aligning with the predicted short isoform of *G. sulfurreducens* PilA, and is missing a region of 19 amino acids found at the N-terminus of the predicted long isoform of *G. sulfurreducens* PilA, which is 90 amino acids long. There is 76% sequence identity between the 60 amino acids of *G. metallireducens* PilA and the

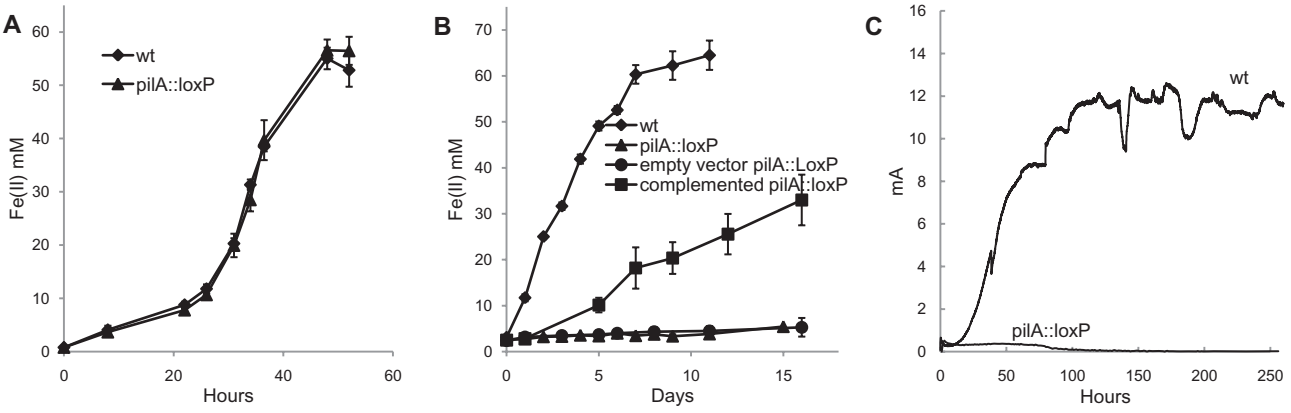

Fig. 4. Reduction of Fe(III) and current production by *G. metallireducens pilA* mutant. *Geobacter metallireducens* wild-type and *ΔpilA::loxP* grown with Fe(III) citrate (A). *Geobacter metallireducens* wild-type, *ΔpilA::loxP* mutant, *pilA* mutant with an empty vector and complemented *pilA* mutant grown with insoluble Fe(III) oxide (B) as the electron acceptor. (C) Current production time-courses of wild-type and *ΔpilA::loxP*. Data are the mean (A and B) or a representative culture (C) of at least three independent experiments.

predicted short isoform of *G. sulfurreducens* PilA, which may account for the apparent similar function.

Implications

The development of a strategy for genetic manipulation of *G. metallireducens* is an important step further in understanding the physiology of the genus *Geobacter*, which plays an important role in anaerobic soils and sediments (Lovley *et al*., 2004). Analysis of the available genome sequences of *Geobacter* species suggests that they may share many common features (Methe *et al*., 2003; Aklujkar *et al*., 2009; 2010; Butler *et al*., 2009; 2010). One example is the unique sequence for the type IV pili found only in members of the *Geobacteraceae* family (Reguera *et al*., 2005). The results presented here indicate that the pili of *G. metallireducens* are important for Fe(III) oxide reduction and electron transfer to electrodes, as previously found for *G. sulfurreducens*. There are also significant differences between *Geobacter* species (Butler *et al*., 2007; 2009; 2010; Aklujkar *et al*., 2009). For example, the results shown here suggest that one of the reasons that *G. metallireducens* may be a more effective Fe(III) oxide reducer than *G. sulfurreducens* is that *G. metallireducens* is motile.

In addition to pili, outer surface *c*-type cytochromes are important for extracellular electron exchange of *G. sulfurreducens* with Fe(III) oxide (Leang *et al*., 2003; Mehta *et al*., 2005), U(VI) (Shelobolina *et al*., 2007), humic substances (Voordeckers *et al*., 2010), electrodes (Holmes *et al*., 2006; Nevin *et al*., 2009) and other cells (Summers *et al*., 2010). However, there is poor conservation of outer surface cytochromes between *G. sulfurreducens* and *G. metallireducens*. Further study of the functional homologues in *G. metallireducens* is likely to provide important insight into the important features that *c*-type cytochromes may share to permit similar function in the absence of sequence homology. Such studies are underway.

Acknowledgements

The work presented herein was funded by the Advanced Research Projects Agency-Energy (ARPA-E), U.S. Department of Energy, under Award Number DE-AR0000087 and DE-AR0000159.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Genotype of the *G. metallireducens ΔfliC::Sp*^{r} mutant. DNA gels showing PCR results using a primer annealing 760 bp upstream of the *fliC* coding sequence and a second primer annealing to the spectinomycin resistance cassette (A) and PCR results using a primer annealing 500 bp upstream of *fliC* and a second primer annealing within the *fliC* coding sequence (B) with potential mutants (lanes 1–4) and the wild-type (lane wt). The numbers on the left indicate the band sizes in kb for the NEB 1 kb ladder used as a marker (lane ladder). Genomic DNA was used as template for PCR reactions.
Fig. S2. Genotype of the *G. metallireducens ΔpilA::loxP* mutant. DNA gels showing PCR results using a primer annealing 600 bp upstream of *pilA* coding sequence and a second primer annealing in the spectinomycin resistance cassette (A) and PCR results using a primer annealing 500 bp upstream of *pilA* and a second primer annealing within the coding sequence of *pilA* (B) with potential *ΔpilA::Sp*^{r}*loxP* mutants (lanes 1–5) and the wild-type (lane wt). (C) DNA gel showing PCR results using primers annealing 500 bp upstream and downstream of the *pilA* coding sequence with *pilA* mutants obtained after the introduction of the Cre recombinase expression plasmid (lanes *ΔpilA::loxP* 1-2), a control mutant before this treatment (lane *ΔpilA::Sp*^{r}*loxP*) and the wild-type (lane wt). The numbers on the left indicate the band sizes in kb for the NEB 1 kb ladder used as a marker (lane ladder). Genomic DNA was used as template for PCR reactions.
Fig. S3. Gene deletion in *G. metallireducens*. Single-step gene replacement of *fliC* (A). A plasmid bearing a construct containing the 500 bp upstream and downstream of the coding sequence (CDS) of *fliC* separated by a spectinomycin resistance cassette was linearized by restriction enzyme digestion. The linearized plasmid was electroporated into *G. metallireducens*. Homologous recombination resulted in the replacement of the *fliC* wild-type allele by the mutant allele. Markerless deletion of *pilA* with the Cre-lox system (B). Single-step gene replacement was used to replace the *pilA* wild-type allele with a mutant allele in which the coding sequence of *pilA* was replaced by a spectinomycin resistance cassette flanked by two *loxP* sites. Introduction of the Cre recombinase resulted in the loss of the spectinomycin resistance cassette by recombination of the two *loxP* sites.
Table S1. Bacterial strains and plasmids used in this study.
Table S2. Primers used for mutant construction and genotype validation.
Appendix S1. Experimental procedures.

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