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

Tremblay, Pier-Luc; Aklujkar, Muktak; Leang, Ching; Nevin, Kelly P.; Lovley, Derek

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

Link back to DTU Orbit

Citation (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
A genetic system for *Geobacter metallireducens*: role of the flagellin and pilin in the reduction of Fe(III) oxide

Pier-Luc Tremblay,* Muktak Aklujkar, Ching Leang, Kelly P. Nevin and Derek Lovley
Department of Microbiology, University of Massachusetts, Amherst, MA, USA.

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 electrotformed 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...
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...
accepator 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% ± 0.1% of total Fe reduced to Fe(II) per day) was much faster than that of the fliC-deficient mutant (0.2% ± 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
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...
predicted short isoform of \textit{G. sulfurreducens} PilA, which may account for the apparent similar function.

**Implications**

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

In addition to pili, outer surface c-type cytochromes are important for extracellular electron exchange of \textit{G. sulfurreducens} with Fe(III) oxide (Leang \textit{et al}., 2003; Mehta \textit{et al}., 2005), U(VI) (Shelobolina \textit{et al}., 2007), humic substances (Voordekers \textit{et al}., 2010), electrodes (Holmes \textit{et al}., 2006; Nevin \textit{et al}., 2009) and other cells (Summers \textit{et al}., 2010). However, there is poor conservation of outer surface cytochromes between \textit{G. sulfurreducens} and \textit{G. metallireducens}. Further study of the functional homologues in \textit{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.

**References**


c-type cytochromes for U(VI) reduction by Geobacter sulfurreducens. BMC Microbiol 7: 16.

Supporting information

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

Fig. S1. Genotype of the G. metallireducens ΔpilA::loxP mutant. DNA gels showing PCR results using a primer annealing 760 bp upstream of the pilA 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'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'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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.