Exploring genetic tools in environmental microbes: Applications in extracellular electron transfer

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Arpita Bose and Zhecheng Zhang explore genetic tools in environmental microbes, citing applications in extracellular electron transfer

This article reviews three main genetic approaches that have expanded our ability to study electroactive bacteria, particularly those that are not traditional model organisms but can perform extracellular electron transfer (EET).

The first approach is CRISPR genome editing, a method that allows for targeted gene knockouts for specific modifications in redox-related pathways.

The second is transposon insertion sequencing (TIS), also known as TIS or RB-TnSeq, which enables high-throughput screening of mutant libraries to identify genes required for respiration on electrodes or mineral surfaces.

The third strategy involves programmable control of gene expression, tools such as broad-host- range plasmids, inducible promoters, or CRISPR interference and activation (CRISPRi and CRISPRa) to adjust the activity of multiple genes within EET pathways. Building on recent studies in S. oneidensis, Geobacter, and other environmental isolates, this review links these genetic tools to a new understanding of cytochromes, pili, and biofilm-mediated electron transfer. Finally, it discusses practical challenges and solutions for applying these methods to environmental bacteria that are still difficult to manipulate genetically.

Why genetic tools?

EET is a microbial process with significant environmental and technological importance. It plays a role in mineral cycling within sediments and supports power generation in microbial fuel cells. Early research identified conductive pili, cytochrome, and redox shuttles as key components that allow bacteria such as Geobacter and Shewanella to transfer electrons beyond the cell boundary.

However, much of this foundational work was based on indirect observations and correlations. Moving from description to engineering of these pathways required precise genetic manipulation to test specific genes, analyze networks, and reprogram microbial function. Recent developments in synthetic biology, including CRISPR-based genome editing, transposon mutagenesis, and plasmid-based expression systems, now provide the necessary precision for these studies.

These genetic tools help researchers determine complex interactions in EET networks, such as parallel electron transport chains or redundant cytochrome families that were previously indistinguishable using biochemical assays, and improve strains for use in bioenergy and bioremediation. Without these methods, our understanding of electroactive microbes and our ability to enhance their functions would remain limited.

Challenges in environmental Isolates

Many environmentally important microbes capable of EET are not model organisms. Their thick cell walls, stress responses, and specific growth requirements make genetic manipulation challenging. For example, species such as Cupriavidus metallidurans CH34 and isolates from anaerobic environments often resist transformation, lack well-characterized promoters or selection markers, and need specialized cultivation methods. These microbes may also have unique codon usage or regulatory systems that are not compatible with standard genetic tools.

Moreover, horizontal gene transfer barriers and strong endogenous restriction-modification systems can degrade foreign DNA before it integrates, making transformation success highly strain specific. In the past, these challenges meant that researchers had to focus on model strains or rely on observational data. Recent advances, including host-adapted CRISPR systems, modular plasmids, and pooled mutagenesis techniques, are beginning to address these barriers. This progress is making it possible to conduct systematic genetic studies even in strains that were previously difficult to work with.

CRISPR genome editing in EET research

CRISPR-Cas9 editing allows direct testing of gene function by enabling deletions, insertions, or modifications at target loci. In C. metallidurans, Kovacs et al. engineered a single-plasmid CRISPR-Cas9 toolkit for markerless deletion of the pilA and piE genes, which are hypothesized to encode conductive pili. Surprisingly, the mutants lacking these genes still produced current, suggesting alternative EET mechanisms.

Similarly, in S. oneidensis, CRISPR-mediated edits to the MtrCAB operon clarified its role in electron transfer across the outer membrane. CRISPR edits to the MtrCAB operon made clear that MtrC and OmcA serve as key outer membrane cytochromes for electron transfer to solid substrates. MtrA was shown to relay electrons from the inner membrane, forming a modular conduit with MtrC. Disrupting these components led to reduced current output, confirming their essential role in EET.

CRISPR also enables multiplexed editing, allowing for the simultaneous modification of multiple genes or pathways to study synthetic interactions and co-dependencies in EET processes. Compared to traditional recombinant methods, CRISPR dramatically reduces modification time and enables multiplexed perturbations – critical for dissecting and understanding complex, overlapping electron transfer systems.

Transposon insertion sequencing (TIS/RB-TnSeq)

TIS methods enable high-throughput discoveries of genes involved in electrode respiration. For instance, Baym and colleagues used a technique called Knockout Sudoku to generate a whole genome transposon library in S. oneidensis, targeting more than 3,600 genes. Screening this library under conditions with electrodes or soluble electron acceptor reveals not only core cytochrome but also periplasmic and regulatory components that are essential for EET.

This approach identifies both known and previously unrecognized contributors to electron flow, such as periplasmic electron relays that connect the inner membrane to outer membrane complexes. Interestingly, some insertions affected genes not annotated as redox-related, highlighting previously overlooked contributors to EET, such as outer membrane structural proteins and secondary metabolite regulators.

TIS enables researchers to examine gene function across the entire genome. By systematically distributing individual genes, TIS measures how each loss influences microbial growth or electron transfer in defined conditions. In contrast to methods that rely on sequence homology, TIS can reveal essential genes that are unannotated or poorly characterized. This information is useful for selecting specific genes to target with knockout strategies or to adjust gene expression using CRISPR-Cas9 or plasmid-based systems.

Programmable expression systems (Plasmids and CRISPRi/a)

Some EET genes cannot be simply knocked out – they require controlled expression. Cao et al. developed a synthetic plasmid toolkit for S. oneidensis that measures promoter strength using GFP reporters and regulates MtrCAB expression. They found that moderate expression, not maximal, produced the highest EET efficiency, showing that EET pathways have expression sweet spots.

For more dynamic control, Chen et al. designed a CRISPR-PAIR system in Shewanella that combines gene repression and activation. Tuning redox enzymes and cytochromes boosted electron output by nearly 4-fold, demonstrating the potential of multiplexed regulation in optimizing bioelectrochemical performance.

These tools also allow temporal and conditional control of gene expression, enabling tests of how gene activity fluctuates with environmental redox potential, oxygen levels, or electrode configuration. Programmable expression systems allow researchers to test gene dosage, timing, and combinations that contribute to optimal EET. These insights are not accessible through gene knockout alone.

Integrating tools and future applications

Used together, these tools create a complete workflow: candidate genes are discovered using TIS, their function is validated through CRISPR knockouts, and gene expression is fine-tuned using plasmids or CRISPRi/a. This layered approach helps clarify complex EET pathways that involve overlapping cytochrome families, pili structures, and metabolic modules.

Applying these tools in combination has already clarified how energy is routed under different environmental conditions, for example, distinguishing direct EET via cytochromes from flavin-mediated shuttling under oxygen-limited conditions. Current priorities include adapting these toolkits for use in anaerobic and multispecies communities, improving transformation methods for strains that are difficult to edit, and developing a modular system that can be introduced into newly isolated microbes from sediments or wastewater. Making microbes easier to modify genetically is essential for building mixed microbial systems or improving bioreactors that rely on electron transfer.

Conclusion

As synthetic biology advances, applying genetic tools to environmental microbes is changing how we study EET. CRISPR, TIS, and programmable expression systems allow for precise investigation of EET genes in a range of species. These developments are moving the field from observation to experimentation and from description to prediction, supporting progress in both basic research and applied bioenergy systems.

Looking ahead, combining genetic tools with omics data, which includes large-scale analyses of genes, proteins, and metabolites, as well as electrochemical measurements, may enable more precise engineering of microbial communities for applications such as energy storage, biosensing, and pollutant remediation. This suggests that the developments of these toolkits will move the field beyond understanding natural microbial processes toward designing systems that achieve specific functional goals.

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