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Extracellular Electron Transfer: Respiratory or nutrient homeostasis?

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ABSTRACT

Exoelectrogens are able to transfer electrons extracellularly, enabling them to respire on insoluble terminal electron acceptors. Extensively studied exoelectrogens like *Geobacter sulfurreducens* and *Shewanella oneindensis* are Gram-negative, but more recently it has been reported that Gram-positive bacteria like *Listeria monocytogenes* and *Enterococcus faecalis* also exhibit the ability to transfer electrons extracellularly, although it is yet unclear whether this has a function in respiration or in redox control of the environment, for instance by reducing ferric iron for iron uptake. In the current issue of *Journal of Bacteriology*, Hederstedt, Gorton and Pankratova report on experiments that directly compare extracellular electron transfer (EET) pathways for ferric iron reduction and respiration and find a clear difference, providing further insights and new questions into the function and metabolic pathways of EET in Gram-positives.

KEYWORDS: extracellular electron transfer, Gram-positive bacteria, ferric iron reduction.

Over the last two decades, exoelectrogens have attracted widespread attention due to their ability to generate electricity in microbial fuel cells (MFCs) or synthesise chemicals in microbial electrosynthesis.(1, 2) In nature, exoelectrogens transfer electrons to insoluble terminal electron acceptors such as iron(III)oxide or manganese(III/IV)oxide minerals. In microbial electrochemical systems, this ability is exploited by replacing the insoluble electron acceptors with a macroscopic conductive electrode, i.e. the anode. Electricity is generated by connecting the anode/biofilm to a cathode where the electrons are consumed, typically by reducing oxygen to water. The electron transfer from the anode to the cathode gives rise to the electrical current of the MFCs. In microbial electrosynthesis, the electron transfer direction is typically reversed and electrons are provided to the microbial community by applying a potential to the electrode/biofilm. MFCs and microbial electrosynthetic systems are generally known as microbial electrochemical systems (MESs). In early MESs, electrons were transferred between the microbial community and the electrode by electron mediators. Mediators are electroactive chemicals, such as ferricyanide, which are added to the medium. These mediators were often toxic, limiting the lifespan of the system, added cost to the MES or limited the power output of MFC. The discovery that exoelectrogens could transfer respiratory electrons 'naturally' to an electrode without the need to add electron mediators solved many of the bottlenecks of the earlier MESs.

Further development of MESs requires, among others, an understanding of the electron transfer pathways that operate inside and around bacteria. Alternative respiratory pathways have been discovered in different organisms and the best characterised pathways are from *Geobacter sulfurreducens* and *Shewanella oneindensis*. *G. sulfurreducens* transfers electron over large distances via conducting pili or filaments. The exact nature, composition and conductivity of these pili and filaments is still heavily debated.(3-5) *S. oneidensis* transfers electrons extracellularly via outer membrane cytochromes, with the MtrCAB complex being the best characterised.(6, 7) Both *G. sulfurreducens* and *S. oneindensis* contain an array of cytochromes in their inner membranes, periplasms and outer membranes and the exact respiratory electron transfer pathway still requires further study. It might be that multiple pathways operate simultaneously.

Exoelectrogens are typically Gram-negative. However, more recently, it has been shown that Gram-positive bacteria can also transfer electrons extracellularly. Gorton and co-workers were one of the first to show that if *Bacillus subtilis* is embedded in an osmium-containing redox polymer, electron transfer between *B. subtilis* and electrode is possible.(8) Electrical current was higher when an engineered strain was used in which the succinate/quinone oxidoreductase (respiratory complex II) is overproduced. This suggested that electrons originated from the electron transport chain of *B. subtilis*. Since this original publication, Gorton, Hederstedt and co-workers showed that both osmium- and quinone-containing redox polymers can facilitate extracellular electron transfer (EET) in Gram-positive bacterium *Enterococcus faecalis*.(9, 10) *E. faecalis* is unable to synthesise heme, and in the absence of heme in the environment*, E. faecalis* has essentially a fermentative metabolism. However, when supplied with heme as a nutrient, *E. faecalis* produces a minimal respiratory chain: consisting of a 'conventional' type-2 NADH dehydrogenase (NDH-2), which reduces demethylmenaquinone (DMK), and a terminal oxidase (cytochrome *bd*), which re-oxidises the DMK pool. NDH-2 are FAD-containing, single polypeptide enzymes that are peripherally associated with the membrane and do not contain transmembrane helices. NDH-2 oxidises NADH but unlike complex I, they do not transfer protons across the membrane and do not contribute to the proton-motive force. The relatively simple electron transfer chain of *E. faecalis* enabled more in-depth studies, which showed that the quinone pool of *E. faecalis* was essential for EET when embedded in redox polymers.(11)

Many Gram-positive bacteria including *E. faecalis*(12) and *Listeria monocytogenes*(13) are known to be able to reduce extracellular ferric iron. Reduction of extracellular ferric iron is believed to be a common strategy to enhance iron bioavailability and iron uptake in both prokaryotes and eukaryotes.(14, 15) This then raises the question whether EET functions in nutrient uptake (e.g. iron bioavailability) at an energy cost or in respiration, aiding in the production of energy. It might also be possible that an EET pathway mainly functioning in a non-respiratory function such as iron uptake, becomes the sole available route to access terminal electron acceptors under laboratory conditions and thereby acquires a respiratory function. To illustrate the complexity of issue, in a ground breaking work by Light *et al*., a genomic characterisation of EET pathways in *L. monocytogenes* was based on an assay that monitored the extracellular reduction of ferric iron.(13) Other functions can also be explored,

such as homeostatic control of, for instance, the NADH/NAD^+ redox balance or the proton-motive force where the EET pathway could act as safety valve or energy spilling mechanism.

The question of nutrient uptake versus respiration is dependent on the actual EET pathway and, crucially, whether the EET pathway contributes to the generation of energy and/or the proton-motive force. Furthermore, it will be dependent on the concentration of ferric iron and other terminal electron acceptors including oxygen. Under iron limited conditions, expressing an EET pathway to uptake a small amount of iron (small with respect to respiration and total metabolism of the cell) has a comparatively high energy cost, and could be classed as a nutrient uptake pathway. When under limitation of terminal electron acceptors, but with replete ferric iron, the reduction of ferric iron could be classed as respiration.

Both *E. faecalis* and *L. monocytogenes* are facultative anaerobes and prevail in low-oxygen environments during infection (urinary tract and intestinal tract, respectively). Interestingly, Light *et al.* showed that the EET pathway is important for survival of *L. monocytogenes* in the intestinal lumen.(13) On the other hand, for internalised cells, the EET pathway is dispensable and instead the electron transport chain pathway (via cytochrome *bd* and aa_3) has been shown obligatory for their survival.(16) More recently, Light *et al*. also identified a divergent extracellular reductase subfamily in *L. monocytogenes*, which indicates a more general role for EET in providing electrons to extracellularly localised reductases.(17) Many of these enzymes have unknown substrate specify, so the question of the functional importance of EET remains unanswered. Nonetheless, and importantly, one extracellular enzyme was identified as a fumarate reductase. As *L. monocytogenes* can respire on fumarate when grown on the sugar xylitol, this finding suggests that the EET pathway also has a primary function in respiration.

The paper from Hederstedt, Gorton and Pankratova in this issue of the *Journal of Bacteriology* uniquely approaches this question by directly comparing ferric iron reduction and EET abilities of *E. faecalis* mutants. Naturally, ferric iron reduction is an extracellular reaction and thus requires EET. To study EET, electron transfer to ferric iron and an osmium-containing redox polymer were compared. As described above, the redox polymer can extract electrons, either directly or indirectly, from the inner membrane quinone pool, oxidising DMK and thereby supporting respiration. Ferric iron reduction in *E. faecalis* was found to be dependent on two membrane associated proteins: A type-2 NADH dehydrogenase Ndh3 (here named ^{Eet}NDH-2) and EetA. The ^{Eet}NDH-2 enzymes from *E. faecalis* and *L. monocytogenes* are homologous and ^{Eet}NDH-2, like EetA, was identified by Light *et al*.(13) to be required for extracellular ferric iron reduction in *L. monocytogenes* (^{Eet}NDH-2 in *L. monocytogenes* is Ndh2 or NDH-2b). This thus confirms that ^{Eet}NDH-2 and EetA constitute a general EET pathway in Gram-positive bacteria that supplies electrons to reductases located extracellularly. It should be stressed here that Hederstedt *et al*. show that when *E. faecalis* is able to respire aerobically on molecular oxygen, EET to ferric iron is attenuated. This is most likely because the terminal oxidase (cytochrome *bd*) competes with ^{Eet}NDH-2 by oxidizing the DMK pool. This could be interpreted as supporting a respiratory role of EET, although it remains to be elucidated whether EET to ferric iron contributes to the proton-motive force.

^{Eet}NDH-2 is not the only NDH-2 in *E. faecalis* or *L. monocytogenes* and it is unclear why EET activity to reduce extracellular ferric iron is only associated with ^{Eet}NDH-2. Light *et al.* suggested that two NDH-2 enzymes in *L. monocytogenes*, i.e. NDH-2 (Ndh1/NDH-2a) and ^{Eet}NDH-2 (Ndh2/NDH-2b), are distinct in that they utilise menaquinone (MK) and DMK, respectively.(13) DMK is a precursor of MK and demethylmenaquinone methyl transferase (MenG) is responsible for addition of a methyl group to the quinone head group of DMK. This extra methyl group lowers the redox potential.(18) However, *E. faecalis* lacks a *menG* gene and only has DMK as main quinone, excluding the possibility that the same distinction plays a role in this organism.(19)

Recent work by us confirmed a NDH-2 in *L. monocytogenes* (Ndh1/NDH-2a) that performs the conventional NDH-2 catalysis, i.e. NADH:menaquinone oxidoreduction.(20) Because one of the *E. faecalis* NDH-2 (Ndh2) is highly homologous to *L. monocytogenes* cNDH-2, it is likely that this *E. faecalis* NDH-2 also catalyses NADH:DMK oxidoreduction and is thus a 'conventional' NDH-2. Indeed, Hederstedt *et al*. showed that, using a membrane extract, NADH oxidation activity was associated with cNDH-2. Then, the question remains how *E. faecalis* and *L. monocytogenes* (and other Gram positive bacteria) utilise multiple NDH-2 enzymes to generate distinctive electron transfer pathways?

One major difference between ^{Eet}NDH-2 enzymes and NDH-2 enzymes is that the ^{Eet}NDH-2 enzymes have an extension of ~200 amino acid residues at the C-terminus and the primary sequence suggests that this domain contains several transmembrane helices. The biochemical and biological function of this extension is currently unknown and difficult to deduce from their amino acid sequences, given this extension is diverse among ^{Eet}NDH-2 enzymes from different organisms.(21) However, as pointed out by Hederstedt *et al.*, *E. faecalis* and *L. monocytogenes* ^{Eet}NDH-2 have highly homologous C-terminal extensions. The ^{Eet}NDH-2 enzymes from *E. faecalis* and *L. monocytogenes* might thus form a specific electron-transfer protein complex with other proteins, to efficiently oxidize quinone and release electrons to extracellular acceptors. Alternatively, in *E. faecalis* and *L. monocytogenes*, other genes associated with an EET pathway (e.g. *eetA*, *eetB* and *dmkB*) are clustered with ^{Eet}*ndh-2* and their gene expression levels might be tightly regulated. Therefore, any mutations around this locus might result in loss of DMK production. The current study by Hederstedt *et al.*, showing disruption at the intergenic region led to the EET deficit phenotypes, might support this idea.

The importance of the work by Hederstedt *et al*. in this issue is found in the distinct EET pathways where electron transfer *via* the osmium-containing redox polymer is independent on ^{Eet}NDH-2 or EetA. In other words, not all EET pathways proceed via ^{Eet}NDH-2/EetA. This opens the possibility that different EET pathways exist with varying degrees of alternative functions in nutrient uptake and respiration. It is obvious that the osmium-containing redox polymers do not constitute a naturally occurring environment condition of *E. faecalis* and hence this pathway might not represent a physiologically relevant situation. However, in the development of MESs, non-native pathways could and should be exploited. A cynic might argue that osmium-containing redox polymers are akin to added electron mediators, which in early-day MFC technology was shown to be problematic due to toxicity, cost and reduced power output. Nonetheless, the work of Hederstedt, Gorton and Pankratova, for the first

time, is able to directly compare EET as studied in microbial electrochemical systems with EET pathways that have a metabolic function and reduce extracellular substrates, either in a respiratory or nutrient-uptake capacity.

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