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Hederstedt, Lars; Gorton, Lo; Pankratova, Galina

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Two routes for extracellular electron transfer in

*Enterococcus faecalis*

Lars Hederstedt [a]*, Lo Gorton [b] and Galina Pankratova [b] [c]

[a] The Microbiology Group, Department of Biology, Lund University, Sölvegatan 35, SE-223
62 Lund, Sweden, and [b] Department of Biochemistry and Structural Biology, Lund University, P.O. Box SE-22100 Lund, Sweden. [c] National Centre of Nano Fabrication and Characterization, DTU Nanolab, Technical University of Denmark, Kongens Lyngby, Denmark.

* Corresponding author. E-mail Lars.Hederstedt@biol.lu.se

ABSTRACT

*Enterococcus faecalis* cells are known to have ferric reductase activity and the ability to transfer electrons generated in metabolism to the external environment. We have isolated mutants defective in ferric reductase activity and studied their electron transfer properties to electrodes mediated by ferric ions and an osmium complex modified redox polymer (OsRP), respectively. Electron transfer mediated with ferric ions and ferric reductase activity were both found dependent on the membrane associated Ndh3 and EetA proteins, consistent with findings in *Listeria monocytogenes*. In contrast, electron transfer mediated with OsRP was independent of these two proteins. Quinone in the cell membrane was required for the electron transfer with both mediators. The combined results demonstrate that extracellular electron transfer from reduced quinone to ferric ions and to OsRP occurs via different routes in the cell envelope of *E. faecalis*.

IMPORTANCE

Transfer of reducing power in the form of electrons, generated in catabolism of nutrients, from a bacterium to an extracellular acceptor appears common in nature. The electron acceptor can be another cell or innate material. Such extracellular electron transfer contributes to syntrophic metabolism and is of wide environmental, industrial, and medical importance. Electron transfer between microorganisms and electrodes is fundamental in microbial fuel cells for energy production and for
electricity-driven synthesis of chemical compounds in cells. In contrast to the much studied extracellular electron transfer mediated by cell surface exposed cytochromes, little is known about components and mechanisms for such electron transfer in organisms without these cytochromes and in gram-positive bacteria, like *E. faecalis* which is a commensal gut lactic acid bacterium and opportunistic pathogen.


**INTRODUCTION**

Enterococci are gram-positive bacteria present as commensals in the intestine of animals and some species are opportunistic pathogens, for example *Enterococcus faecalis* and *E. faecium*. The enterococci are lactic acid bacteria phylogenetically closely related to common human pathogens, for example streptococci and pneumococci, and to bacteria of large importance for the food industry, for example lactococci. Enterococci are also found in other environments, such as in consortia of microbes in microbial fuel cells where energy in the form of electricity is harvested by wiring metabolism of electroactive microbes to electrodes (1, 2). With *E. faecalis* cells we have demonstrated and characterized extracellular electron transfer (EET) mediated by various mediators on graphite and gold electrodes (3, 4).

Electricigenicity seems to be a physiological property of many microorganisms (5). Recent studies have revealed EET in the mammalian gut and identified other electroactive gut bacteria (6-8). Knowledge about the mechanisms behind EET in bacteria is not only of clinical relevance but very important for biotechnical applications such as in the design of microbial fuel cells. The nature of electron transfer pathways in electrode-bacterium interaction remains the primary issue in microbial electrochemical techniques, cf (2, 9, 10).

Being a lactic acid bacterium with a mainly fermentative metabolism *E. faecalis* grows well also under anoxic conditions. If supplied with heme the bacterium can respire with molecular oxygen due to the assembly of a membrane bound cytochrome *bd*, which is a terminal quinol oxidase reducing molecular oxygen to water (11, 12). *E. faecalis* contains demethylmenaquinone (DMK) as the only quinone in the membrane. Electrons generated in glycolysis in the cytoplasm are via the activity of
membrane associated dehydrogenases, for example NADH dehydrogenase, mediated to reduce DMK which is oxidized by cytochrome bd or by EET processes. We have demonstrated that DMK is essential for EET and that cytochrome bd activity attenuates EET efficiency in *E. faecalis* (4). Effective electron transfer from the bacterial cell to an electrode requires a mediator – either a highly flexible redox polymer that forms a hydrogel together with the bacterial cells and precipitates onto the electrode surface, i.e., osmium-containing redox polymer ([Os(2,2′-bipyridine)2-poly(N-vinylimidazole) 10Cl]2+/+) (OsRP), or an added diffusible compound such as ferricyanide (1, 3, 4).

*E. faecalis* and several other *Enterococcus* species can reduce exogenous ferric ions (13-16). This dissimilatory activity apparently occurs as an electron sink to oxidize accumulating intracellular reducing equivalents, e.g., NADH, and the activity influences the yield and composition of carbon containing end products obtained from catabolism of, e.g., glucose (14). Research with *Listeria monocytogenes* has revealed a single gene cluster on the chromosome important for both ferric reductase activity and EET (13). Genes in the cluster encode a type II NADH dehydrogenase, a flavoprotein (PplA), two small proteins (EetA and EetB), and quinone synthesis enzymes. A similar gene cluster is present in many other gram-positive bacteria, including *E. faecalis* (13) (Fig 1). Studies with *E. faecalis* have shown that ferric ion metabolism is associated with biofilm formation and identified genes important for this property, but these genes are not orthologous to those found important for ferric reductase activity in *L. monocytogenes* (16). These recent findings together prompted us to investigate to what extent EET to electrodes and ferric reductase activity are connected processes in *E. faecalis*. While our research work was in progress it was reported that EET to ferric iron in *E. faecalis* depends on genes corresponding to those in the gene cluster originally found in *L. monocytogenes* and on a gene encoding the tip adhesion protein EbpA (17). To identify proteins involved in EET to ferric ions and determine if these also are crucial for EET we have here screened a collection of mutants for ferric reductase deficiency, identified the responsible mutations, and analyzed bioelectrochemical properties of the mutants.

**RESULTS**

*Requirements for ferric reductase activity of E. faecalis cells*
Ferric reductase activity of washed *E. faecalis* cells in growth medium supplemented with glucose was determined with ferric sulfate and ferricyanide as alternative substrates as described in Methods and Materials. *E. faecalis* cells cannot synthesize heme, but encode two heme proteins, cytochrome *bd* and catalase (12). To determine the effect of heme and cytochrome *bd* on the ferric reductase activity, the laboratory model strain OG1RF (Table 1) was grown in TSBG with and without hemin added. Heme in the growth medium caused attenuation of ferric reductase activity (Table 2), most likely because cytochrome *bd* oxidase activity decreases the reduction level of the DMK pool in the cytoplasmic membrane (4). Ferric reductase activity was found dependent on DMK in the cell membrane, i.e., strain WY84 which lacks DMK showed low (~15%) activity compared to the parental strain grown under the same conditions (Table 2 and Fig. 2). These results indicated that reduction of ferric ions on the outside of *E. faecalis* cells is mediated by reduced DMK.

**Identification of genes important for ferric reductase activity.**

The genes *ndh2* and *pplA* encoding the type II NADH dehydrogenase Ndh2 and the flavoprotein PplA, respectively, have in *L. monocytogenes* been shown important for ferric reductase activity. We therefore analyzed ferric reductase activity of *E. faecalis* strains with the corresponding genes, *ndh3* and *pplA*, respectively, inactivated (Table 1). The *ndh3* mutant (*ndh3::Tn*) showed low activity with both ammonium ferric sulfate and ferricyanide (Fig. 2). That with *pplA* inactivated (*pplA::Tn*) showed slightly reduced activity with ammonium ferric sulfate and increased activity with ferricyanide (Fig. 2).

To find additional genes important for ferric reductase activity in *E. faecalis* we subsequently screened a library containing clones with random transposon insertion in the chromosome for defective ferric reductase activity. The library was previously constructed in our laboratory using *E. faecalis* OG1RF and the mini mariner transposon (EfaMarTn) (18). Clones of the library in 96 wells format were spotted on agar plates containing growth medium supplemented with 0.2 mM ammonium ferric sulfate and screened for ferric reductase activity using an agar plate colony overlay staining procedure developed for *L. monocytogenes* (19) and adjusted for *E. faecalis* (see Methods and Materials).
Five ferric reductase defective mutants (LFR1, -9, -15, -16 and -19) were identified among ~2000 screened clones (Table 1). The mutant cells grown in TSBG showed low ferric reductase activity compared to the parental strain (Fig. 2). This low activity was similar to that of parental cells grown in the presence of hemin (Table 2) and to that of strain WY84 lacking DMK (Fig. 2). Thus, a residual ferric reductase activity remained in both the isolated mutants and in strain WY84.

The insertion site for EfaMarTn in the chromosome of each mutant was determined by the use of inverse PCR with transposon-specific primers, PCR with region-specific primers (Table S2), combined with DNA sequence analysis. Three mutants (LFR15, -16 and -19) were found to have the ndh3 gene disrupted by insertion of the transposon, one (LFR9) had the insertion in the eetA gene, and one (LFR1) had it in the ndh3-eetA intergenic region (Fig. 1 and Table 1).

**Properties of mutants defective in ndh3 or eetA**

Isolated membranes of the ferric reductase defective mutants grown in the presence of heme, to generate an active cytochrome bd in the cells, showed ≥43 % NADH oxidase activity compared to the parental strain OG1RF (Table 3). This rather high membrane bound NADH oxidase activity remaining in Ndh3 defective mutants can be explained by the presence of two other type II NADH dehydrogenases in *E. faecalis*: Ndh and Ndh2. As expected, the DMK deficient strain WY84 and all strains grown in the absence of hemin, lacked NADH oxidase activity because of the absence of cytochrome bd activity (Table 3). The results show that Ndh3 is required for ferric reductase activity but contributes only marginally to respiration with molecular oxygen. The colony size on agar plates and the growth of ferric reductase defective mutants in liquid medium (TSBG with and without hemin) were similar to that of the parental strain OG1RF, indicating no gross defect in metabolism in the mutants.

**Electrochemical characterization of mutants**

It has been reported that ferric reductase activity in *L. monocytogenes* is important for EET (13) and that *E. faecalis* biofilm production depends on the environmental iron ion level and on EET (16). We therefore analyzed the electrochemical properties of *E. faecalis* cells defective in ferric reductase activity to perform EET to a graphite electrode in response to added glucose. Cells were grown without heme and the
analysis was done with washed cells that do not multiply during the experiment but are metabolically active. As we have demonstrated before (3, 4), effective electron transfer from *E. faecalis* cells to an electrode requires an artificial redox mediator - either a polymeric or a monomeric shuttle. In the current work we used redox mediators with similar redox potentials (Fig. S1): OsRP (420 ± 4 mV) co-immobilized on the electrode and freely diffusible ferricyanide (426 ± 3 mV), respectively.

Mutants defective in *pplA*, *ndh3*, and *eetA*, respectively, and mediated by OsRP showed electron transfer ability to the electrodes in response to glucose (Fig. 3A). Compared to the parental strain OG1RF, the *ndh3* mutants were slightly affected in EET (Fig. 3A). The *pplA* mutant showed a high activity (Fig. 3B). With ferricyanide as mediator, in contrast, the *ndh3* and *eetA* defective mutants were found to essentially lack EET activity (<5 µA cm⁻¹) (Figs. 3C and S2) while the parental strain and the *pplA* defective mutant showed current densities of >35 µA cm⁻¹ (Fig. 3D) consistent with high ferricyanide reductase activity (Fig. 2). EET with both OsRP and ferricyanide as mediator was strictly dependent on the presence of DMK in the cell as demonstrated using strain WY84 which is blocked in quinone synthesis (Fig. 3B and D).

The results from the electrochemical experiments show that Ndh3- and EetA-dependent ferric reductase activity of *E. faecalis* cells are important for EET with ferric ions as redox mediator. However, with OsRP as mediator Ndh3 and EetA are not involved in the electron transfer from reduced DMK in the cell to the electrode.

**DISCUSSION**

In this work we have among a collection of *E. faecalis* clones with random transposon insertions in the chromosome screened for mutants defective in ferric reductase activity. The isolated mutants were investigated for their ability to transfer electrons generated in glucose catabolism to a graphite electrode depending on type of provided redox mediator. In parallel to our work, Lam et al (17) have studied properties of *E. faecalis* strains with transposon insertion in genes corresponding to those known to be important for ferric reductase activity in *L. monocytogenes* (13) and for DMK synthesis in *E. faecalis*. Although the approaches in our research groups were different and EET was not measured using the same type of
experimental setup, ndh3 (OG1RF_12510), eetA (OG1RF_12511) and menB (OG1RF_10330) were in both studies identified as important for EET. Lam et al in addition showed that eetB (OG1RF_12512), dmkA (OG1RF_12507), and menE (OG1RF_10331) are of major importance for EET (i.e., defective mutants showed < 50% cumulative charge production) (17).

MenB, MenE and DmkA catalyze steps in DMK synthesis from chorismate. EetA (15.8 kDa) is a predicted membrane protein anchored to the outer side of the cytoplasmic membrane by a non-cleaved N-terminal signal sequence. EetB (20.5 kDa) is an integral membrane protein with 4 predicted transmembrane segments and a large periplasmic loop connecting transmembrane segments 3 and 4. EetA and EetB might form a complex in the membrane. Ndh3 (71.8 kDa) and the paralogs Ndh2 (49.7 kDa) and Ndh2 (44.0 kDa) are from the predicted amino acid sequences suggested to be type II NADH:quinone oxidoreductases. Ndh3 is atypical for this class of enzymes by having a C-terminal extension of the polypeptide containing two predicted transmembrane segments. Ndh3 is not important for aerobic respiration and seems specialized for ferric reductase activity in E. faecalis (this work) and in L. monocytogenes, (where the orthologue is Ndh2) (13). Streptococcus agalactiae (a lactic acid bacterium closely related to E. faecalis) contains only one type II NADH dehydrogenase. The gene for this protein is in the same operon as the cydABCD genes encoding cytochrome bd polypeptides. In E. faecalis the ndh2 gene is on the chromosome co-localized with cydABCD and the sequence of Ndh2 is very similar to that of S. agalactiae NADH dehydrogenase. Based on these arguments, Ndh2 is probably the major respiratory NADH dehydrogenase in E. faecalis.

The collected data for E. faecalis (this work and (17)) and L. monocytogenes (13) indicate that an atypical NADH dehydrogenase (Ndh3 in E. faecalis) with the FAD containing domain presumably on the cytoplasmic side of the membrane, EetA which is mainly exposed to the periplasm, and EetB in the membrane, are required for ferric reductase activity dependent on DMK. The basic role of Ndh3 is probably to couple the oxidation of NADH in the cytoplasm to reduction of a quinone species that might be special and synthesized with the help of DmkAB, as suggested by Light et al (13). The function of Ndh3 seems specifically connected to ferric ion reduction involving EetA and EetB. In the presence of active cytochrome bd oxidase and under oxic conditions,
conditions the reduction level of the quinone pool in the membrane of the *E. faecalis* cell is low and this attenuates both ferric reductase activity (Table 2) and EET (4). Thus, cytochrome *bd* is apparently more efficient than the ferric reductase system in oxidizing quinol. Alternatively the Ndh3 enzyme might show low NADH dehydrogenase activity when the NADH/NAD⁺ ratio is low in the cytoplasm. Since, Ndh3 only marginally contributes to NADH oxidase activity (Table 3) it seems like the quinol generated by the activity of Ndh3 is reserved for the reduction of ferric ions via EetAB. The phenotype of *ndh3* defective mutants could alternatively be explained by that the Ndh3 protein via its transmembrane C-terminal extension binds EetA and EetB in an, for function, obligatory protein complex. The role(s) of EetA and EetB in electron transfer from reduced quinone to ferric ion and the actual site of ferric ion reduction in the cell envelope remain enigmatic. It has, however, been shown that the EbpA pilin of the Ebp fiber, which is covalently bound to the peptidoglycan of the cell wall, can bind iron and it is suggested that the Ebp fiber is involved in iron acquisition in *E. faecalis* and might play some role in ferric ion reduction (17).

Electron conducting redox polymers, such as OsRP, are of large potential in bioelectrochemical applications within environmental monitoring and energy transformation. As demonstrated in this work Ndh3 and EetA are not important for electron transfer from the quinol pool to OsRP (Fig. 3). OsRP apparently accepts electrons directly from reduced DMK. Variants of redox hydrogel containing covalently bound quinone can substitute for the lack of DMK in *E. faecalis* cells and mediate glucose-dependent EET (20, 21). In conclusion, function of OsRP as mediator in EET does not rely on components involved in EET mediated by ferric ions. I.e., electron transfer mediated to an electrode by OsRP occurs via a route distinct from that mediated with ferric ions. This aspect of material-cell interface is directly applicable in the development and optimization of microbial electrochemical systems to achieve efficient energy conversion designs.

**MATERIALS AND METHODS**

**Growth of bacteria**

Bacterial strains used in this work are listed in Table 1. *E. faecalis* strains were grown in tryptic soy broth without dextrose (Difco) (TSB) with 1% (w/v) glucose added...
Isolation of ferric reductase mutants
An available collection of _E. faecalis_ OG1RF EfaMarTn insertion mutants (18), stored frozen as individual clones in 20% (w/v) glycerol in 96 well micro titer plates, was replicated to Todd-Hewitt agar plates supplemented with 0.2 mM ammonium ferrisulfate dodeca hydrate. The plates were incubated at 37 °C for one or two days to obtain colonies. Then the agar plates were overlaid with soft agar (0.8% w/v agar) containing 0.5 % glucose and 2 mM FerroZine™ (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´, 4´´-disulfonic acid monosodium salt hydrate). Ferric reductase activity was observed as pink coloration of colonies. Poorly staining colonies were immediately picked and streaked on the same type of plates and the phenotype was confirmed first by repetition of the ferric reductase activity staining of colonies and subsequently by ferric reductase activity determination with washed cells grown in liquid medium.

Identification of EfaMarTn insertions sites in chromosomal DNA
The insertion site of the 2.1 kb transposon EfaMarTn (22) in the chromosome of _E. faecalis_ strain LFR9 and LFR16 was determined using inverse PCR after cleavage of DNA with HindIII and exploiting primers invCATR2 and invGFPR1 for both DNA amplification and sequence analysis, as described before (18). The transposon insertion site in strains LFR1, LFR15 and LFR19 were determined by sequence analysis of PCR products obtained using primers ndh3up and ndh3dw. The genotype of strains ndh3::Tn and pplA::Tn were confirmed using primers ndh3up, ndh3dwn, pplAup, pplAmd, invCATR2 and invGFPR1. Phusion DNA polymerase (Thermo Scientific) was used for PCR. Sequence of primers are presented in Table S1.

Ferric reductase activity assay of cells
Cells were grown in 25 ml TSBG in a 250 ml Erlenmeyer flask at 200 rpm and 37 °C to mid exponential growth phase OD_{600} = 0.6 – 0.8 (90 – 180 min). After harvest of the cells by centrifugation, 2 700 x g for 10 min at room temperature, and one wash in 5 ml of TSB they were suspended in TSB to OD_{600} = 1.2 and stored on ice and analyzed the same day. Ferric reductase activity assayed using FerroZine™ was determined at 37 °C in 0.92 ml in a 1ml cuvette containing TSB, 0.4% (w/v) glucose,
2 mM ammonium ferric sulfate, 1 mM FerroZine™, and cells of OD₆₀₀ 0.4. The assay was started by the addition of 500 µl 2 mM ammonium ferric sulfate in TSB to the cuvette containing the other ingredients. Formation of ferrous-FerroZine™ complex was monitored at 562 nm. Reductase activity was calculated based on the initial rate of increase in absorbance, after subtraction of background value obtained in the absence of Ferrozine™, and using the extinction coefficient 27.9 mM⁻¹ cm⁻¹ (19).

Activity with potassium ferricyanide as the substrate was determined at 37 °C in 1 ml in a 1 ml cuvette containing 35 mM Tris/HCl pH 7.5, 0.4% (w/v) glucose, 1 mM K₃Fe(CN)₆ and cells of OD₆₀₀ 0.4. Two cuvettes and a double beam spectrophotometer was used. Ferricyanide (40 µl 25 mM) was added only to the sample cuvette. This started the reaction which was monitored as reduction in absorption at 420 nm over 2 min. Activity was calculated based on the extinction coefficient 1.04 mM⁻¹ cm⁻¹.

Ferric reductase activities were determined using at least duplicate cell samples grown independently and two or more assays were done per sample.

**Electrochemical measurements**

*E. faecalis* cells were grown in 10 ml of TSBG, 37 °C, overnight. The cells were harvested by centrifugation and washed in 50 mM phosphate buffer. The washed cell suspension was adjusted with the buffer to a cell density of 1 g of wet weight per ml and used in electrochemical experiments.

Electrochemical measurements were done at room temperature in phosphate buffer (50 mM Na₂HPO₄/NaH₂PO₄, 100 mM KCl), pH 7.40 using a three-electrode system. When indicated 0.5 mM potassium ferricyanide, added to the buffer, or osmium complex modified redox polymer (OsRP) [Os(2,2'-bipyridine)₂-poly(N-vinylimidazole)₁₀Cl]²⁺/⁻ (23) with a formal potential of 420 mV vs. the standard hydrogen electrode (SHE), 10 mg mL⁻¹ in water, was applied as a mediators. Spectrographic graphite rods (Alfa Aesar GmbH & Co., ø 3.05 mm) were prepared and modified with *E. faecalis* cells and OsRP as described in (4) and used as working electrodes.
Amperometric measurements were performed at a constant potential of 588 mV versus the standard hydrogen electrode (SHE) controlled by a potentiostat (Zäta Electronik, Höör, Sweden) and using a wall jet electrochemical flow cell with a connected working graphite, platinum counter and Ag/AgCl (0.1 M KCl) reference electrode. All potentials in this work are given in reference to SHE. The electrolyte solution was pumped through the system at a flow rate of 0.5 mL min\(^{-1}\).

Cyclic voltammetry was realized using a stationary solution three-electrode electrochemical cell including a working graphite, a platinum counter and Ag/AgCl (sat. KCl) reference electrode controlled by a potentiostat (AUTOLAB PGSTAT 30, Eco Chemie, Utrecht, The Netherlands).

**Miscellaneous methods**

Isolation of membranes from *E. faecalis* strains and protein determination were done as described before (4). NADH oxidase activity of isolated membranes at +30\(^\circ\) C in aerated 50 mM potassium phosphate buffer, pH 7.4, with an initial NADH concentration of 0.16 mM was determined using an upgraded (OLIS Instruments) Aminco DW-2 spectrophotometer (absorption at 340 nm versus 400 nm) and a 3 ml cuvette with magnetic bar stirrer.

**Acknowledgements**

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**References**


**Table 1.** *E. faecalis* strains used in this work.

<table>
<thead>
<tr>
<th>Strain</th>
<th>EfaMarTn insertion position</th>
<th>Locus tag</th>
<th>Gene</th>
<th>Source of strain/reference</th>
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<td>OG1RF</td>
<td>- a</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(24)</td>
</tr>
<tr>
<td>LFR1</td>
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<td>n.a.</td>
<td>intergenic <em>(ndh3-eetA)</em></td>
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<td>LFR9</td>
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<td><em>eetA</em></td>
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<td><em>ndh3</em></td>
<td>This work</td>
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<tr>
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<tr>
<td>pplA::Tn</td>
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<td>WY84</td>
<td>n.a. b</td>
<td>OG1RF_10330</td>
<td><em>menB</em></td>
<td>(26)</td>
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* OG1RF is the parental strain with no transposon insertion.
* b This strain has the *menB* gene deleted and contains no transposon insertion.
Table 2. Ferric reductase activity of *E. faecalis* cells grown in TSBG with and without hemin supplementation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Hemin (8 µM) added to the growth medium</th>
<th>Ammonium ferric sulfate reductase activity (%)</th>
<th>Ferricyanide reductase activity (%)</th>
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<td></td>
<td></td>
<td>+</td>
<td>10 ±3</td>
<td>13 ±7</td>
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<tr>
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<td>menB</td>
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<td>15 ±4</td>
<td>13 ±8</td>
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<td></td>
<td></td>
<td>+</td>
<td>11 ±7</td>
<td>18 ±6</td>
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</table>

Table 3. NADH oxidase activity of isolated membranes from *E. faecalis* strains grown in TSBG with or without hemin supplementation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene inactivated</th>
<th>Hemin added to the growth medium a</th>
<th>NADH oxidase activity (%) b</th>
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<td>ndh3::Tn</td>
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<td>+</td>
<td>75 ±11</td>
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<td></td>
<td></td>
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<td>&lt; 8</td>
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<tr>
<td>pplA::Tn</td>
<td>pplA</td>
<td>+</td>
<td>126 ±6</td>
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<td>-</td>
<td>&lt; 8</td>
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<tr>
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<td>menB</td>
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<td>&lt; 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>&lt; 8</td>
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<td>ndh3 eetA</td>
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</table>

a 2 µM hemin
b 100% activity was 0.53 nmol NADH oxidized per minute and mg of protein.
Figure 1. Map of the ndh3 gene region in the chromosome of *E. faecalis*. Transposon insertion positions in the five ferric reductase mutants isolated in this work are indicated with vertical arrows. For more information see Table 1.
Figure 2. Ferric reductase activity of *E. faecalis* parental (OG1RF) and mutant strains grown without heme. The strains are described in Table 1. Panel A shows activity with ammonium ferric sulfate. Panel B shows activity with potassium ferriyride. Error bars show SEM based on more than four measurements with at least two biological replicates.
Figure 3. Electrochemical communication between heme-free *E. faecalis* parental (OG1RF) and mutant strains and graphite electrodes in the presence of various D-glucose concentrations. The strains are described in Table 1. Panels A and B show current density responses with cells immobilized on OsRP-coated graphite electrodes. Panels C and D show results for the cells immobilized on graphite electrodes and with ferricyanide (Fe$^{3+}$) as redox mediator. The electrochemical behavior of the mutant strains presented in panel C is in Figure S2 shown with an expanded current density scale.