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Two routes for extracellular electron transfer in

Enterococcus faecalis

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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.

KEYWORDS extracellular electron transfer, ferric reductase, EetA, PplA, *Enterococcus faecalis*, type 2 NADH dehydrogenase.

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

68 membrane associated dehydrogenases, for example NADH dehydrogenase,
69 mediated to reduce DMK which is oxidized by cytochrome *bd* or by EET processes.
70 We have demonstrated that DMK is essential for EET and that cytochrome *bd* activity
71 attenuates EET efficiency in *E. faecalis* (4). Effective electron transfer from the
72 bacterial cell to an electrode requires a mediator – either a highly flexible redox
73 polymer that forms a hydrogel together with the bacterial cells and precipitates onto
74 the electrode surface, i.e., osmium-containing redox polymer ([Os(2,2'-bipyridine)₂-
75 poly(N-vinylimidazole) 10Cl]^{2+/+}) (OsRP), or an added diffusible compound such as
76 ferricyanide (1, 3, 4).

77
78 *E. faecalis* and several other *Enterococcus* species can reduce exogenous ferric ions
79 (13-16). This dissimilatory activity apparently occurs as an electron sink to oxidize
80 accumulating intracellular reducing equivalents, e.g., NADH, and the activity
81 influences the yield and composition of carbon containing end products obtained
82 from catabolism of, e.g., glucose (14). Research with *Listeria monocytogenes* has
83 revealed a single gene cluster on the chromosome important for both ferric reductase
84 activity and EET (13). Genes in the cluster encode a type II NADH dehydrogenase, a
85 flavoprotein (PplA), two small proteins (EetA and EetB), and quinone synthesis
86 enzymes. A similar gene cluster is present in many other gram-positive bacteria,
87 including *E. faecalis* (13) (Fig 1). Studies with *E. faecalis* have shown that ferric ion
88 metabolism is associated with biofilm formation and identified genes important for this
89 property, but these genes are not orthologous to those found important for ferric
90 reductase activity in *L. monocytogenes* (16). These recent findings together
91 prompted us to investigate to what extent EET to electrodes and ferric reductase
92 activity are connected processes in *E. faecalis*. While our research work was in
93 progress it was reported that EET to ferric iron in *E. faecalis* depends on genes
94 corresponding to those in the gene cluster originally found in *L. monocytogenes* and
95 on a gene encoding the tip adhesion protein EbpA (17). To identify proteins involved
96 in EET to ferric ions and determine if these also are crucial for EET we have here
97 screened a collection of mutants for ferric reductase deficiency, identified the
98 responsible mutations, and analyzed bioelectrochemical properties of the mutants.

100 RESULTS

101 *Requirements for ferric reductase activity of E. faecalis cells*

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

115

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

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

125

126 To find additional genes important for ferric reductase activity in *E. faecalis* we
127 subsequently screened a library containing clones with random transposon insertion
128 in the chromosome for defective ferric reductase activity. The library was previously
129 constructed in our laboratory using *E. faecalis* OG1RF and the mini mariner
130 transposon (EfaMarTn) (18). Clones of the library in 96 wells format were spotted on
131 agar plates containing growth medium supplemented with 0.2 mM ammonium ferric
132 sulfate and screened for ferric reductase activity using an agar plate colony overlay
133 staining procedure developed for *L. monocytogenes* (19) and adjusted for *E. faecalis*
134 (see Methods and Materials).

135

136 Five ferric reductase defective mutants (LFR1, -9, -15, -16 and -19) were identified
137 among ~2000 screened clones (Table 1). The mutant cells grown in TSBG showed
138 low ferric reductase activity compared to the parental strain (Fig. 2). This low activity
139 was similar to that of parental cells grown in the presence of hemin (Table 2) and to
140 that of strain WY84 lacking DMK (Fig. 2). Thus, a residual ferric reductase activity
141 remained in both the isolated mutants and in strain WY84.

142

143 The insertion site for EfaMarTn in the chromosome of each mutant was determined
144 by the use of inverse PCR with transposon-specific primers, PCR with region-specific
145 primers (Table S2), combined with DNA sequence analysis. Three mutants (LFR15, -
146 16 and -19) were found to have the *ndh3* gene disrupted by insertion of the
147 transposon, one (LFR9) had the insertion in the *eetA* gene, and one (LFR1) had it in
148 the *ndh3-eetA* intergenic region (Fig. 1 and Table 1).

149

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

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

163

164 **Electrochemical characterization of mutants**

165 It has been reported that ferric reductase activity in *L. monocytogenes* is important for
166 EET (13) and that *E. faecalis* biofilm production depends on the environmental iron
167 ion level and on EET (16). We therefore analyzed the electrochemical properties of
168 *E. faecalis* cells defective in ferric reductase activity to perform EET to a graphite
169 electrode in response to added glucose. Cells were grown without heme and the

170 analysis was done with washed cells that do not multiply during the experiment but
171 are metabolically active. As we have demonstrated before (3, 4), effective electron
172 transfer from *E. faecalis* cells to an electrode requires an artificial redox mediator -
173 either a polymeric or a monomeric shuttle. In the current work we used redox
174 mediators with similar redox potentials (Fig. S1): OsRP (420 ± 4 mV) co-immobilized
175 on the electrode and freely diffusible ferricyanide (426 ± 3 mV), respectively.

176
177 Mutants defective in *pplA*, *ndh3*, and *eetA*, respectively, and mediated by OsRP
178 showed electron transfer ability to the electrodes in response to glucose (Fig. 3A).
179 Compared to the parental strain OG1RF, the *ndh3* mutants were slightly affected in
180 EET (Fig. 3A). The *pplA* mutant showed a high activity (Fig. 3B). With ferricyanide as
181 mediator, in contrast, the *ndh3* and *eetA* defective mutants were found to essentially
182 lack EET activity ($<5 \mu\text{A cm}^{-1}$) (Figs. 3C and S2) while the parental strain and the
183 *pplA* defective mutant showed current densities of $>35 \mu\text{A cm}^{-1}$ (Fig. 3D) consistent
184 with high ferricyanide reductase activity (Fig. 2). EET with both OsRP and
185 ferricyanide as mediator was strictly dependent on the presence of DMK in the cell as
186 demonstrated using strain WY84 which is blocked in quinone synthesis (Fig. 3 B and
187 D).

188
189 The results from the electrochemical experiments show that Ndh3- and EetA-
190 dependent ferric reductase activity of *E. faecalis* cells are important for EET with
191 ferric ions as redox mediator. However, with OsRP as mediator Ndh3 and EetA are
192 not involved in the electron transfer from reduced DMK in the cell to the electrode.

193 194 DISCUSSION

195 In this work we have among a collection of *E. faecalis* clones with random transposon
196 insertions in the chromosome screened for mutants defective in ferric reductase
197 activity. The isolated mutants were investigated for their ability to transfer electrons
198 generated in glucose catabolism to a graphite electrode depending on type of
199 provided redox mediator. In parallel to our work, Lam et al (17) have studied
200 properties of *E. faecalis* strains with transposon insertion in genes corresponding to
201 those known to be important for ferric reductase activity in *L. monocytogenes* (13)
202 and for DMK synthesis in *E. faecalis*. Although the approaches in our research
203 groups were different and EET was not measured using the same type of

204 experimental setup, *ndh3* (OG1RF_12510), *eetA* (OG1RF_12511) and *menB*
205 (OG1RF_10330) were in both studies identified as important for EET. Lam et al in
206 addition showed that *eetB* (OG1RF_12512), *dmkA* (OG1RF_12507, and *menE*
207 (OG1RF_10331) are of major importance for EET (i.e., defective mutants showed <
208 50% cumulative charge production) (17).

209

210 MenB, MenE and DmkA catalyze steps in DMK synthesis from chorismate. EetA
211 (15.8 kDa) is a predicted membrane protein anchored to the outer side of the
212 cytoplasmic membrane by a non-cleaved N-terminal signal sequence. EetB (20.5
213 kDa) is an integral membrane protein with 4 predicted transmembrane segments and
214 a large periplasmic loop connecting transmembrane segments 3 and 4. EetA and
215 EetB might form a complex in the membrane. Ndh3 (71.8 kDa) and the paralogs Ndh
216 (49.7 kDa) and Ndh2 (44.0 kDa) are from the predicted amino acid sequences
217 suggested to be type II NADH:quinone oxidoreductases. Ndh3 is atypical for this
218 class of enzymes by having a C-terminal extension of the polypeptide containing two
219 predicted transmembrane segments. Ndh3 is not important for aerobic respiration
220 and seems specialized for ferric reductase activity in *E. faecalis* (this work) and in *L.*
221 *monocytogenes*, (where the orthologue is Ndh2) (13). *Streptococcus agalactiae* (a
222 lactic acid bacterium closely related to *E. faecalis*) contains only one type II NADH
223 dehydrogenase. The gene for this protein is in the same operon as the *cydABCD*
224 genes encoding cytochrome *bd* polypeptides. In *E. faecalis* the *ndh2* gene is on the
225 chromosome co-localized with *cydABCD* and the sequence of Ndh2 is very similar to
226 that of *S. agalactiae* NADH dehydrogenase. Based on these arguments, Ndh2 is
227 probably the major respiratory NADH dehydrogenase in *E. faecalis*.

228

229 The collected data for *E. faecalis* (this work and (17)) and *L. monocytogenes* (13)
230 indicate that an atypical NADH dehydrogenase (Ndh3 in *E. faecalis*) with the FAD
231 containing domain presumably on the cytoplasmic side of the membrane, EetA which
232 is mainly exposed to the periplasm, and EetB in the membrane, are required for ferric
233 reductase activity dependent on DMK. The basic role of Ndh3 is probably to couple
234 the oxidation of NADH in the cytoplasm to reduction of a quinone species that might
235 be special and synthesized with the help of DmkAB, as suggested by Light et al (13).
236 The function of Ndh3 seems specifically connected to ferric ion reduction involving
237 EetA and EetB. In the presence of active cytochrome *bd* oxidase and under oxic

238 conditions the reduction level of the quinone pool in the membrane of the *E. faecalis*
239 cell is low and this attenuates both ferric reductase activity (Table 2) and EET (4).
240 Thus, cytochrome *bd* is apparently more efficient than the ferric reductase system in
241 oxidizing quinol. Alternatively the Ndh3 enzyme might show low NADH
242 dehydrogenase activity when the NADH/NAD⁺ ratio is low in the cytoplasm. Since,
243 Ndh3 only marginally contributes to NADH oxidase activity (Table 3) it seems like the
244 quinol generated by the activity of Ndh3 is reserved for the reduction of ferric ions via
245 EetAB. The phenotype of *ndh3* defective mutants could alternatively be explained by
246 that the Ndh3 protein via its transmembrane C-terminal extension binds EetA and
247 EetB in an, for function, obligatory protein complex. The role(s) of EetA and EetB in
248 electron transfer from reduced quinone to ferric ion and the actual site of ferric ion
249 reduction in the cell envelope remain enigmatic. It has, however, been shown that the
250 EbpA pilin of the Ebp fiber, which is covalently bound to the peptidoglycan of the cell
251 wall, can bind iron and it is suggested that the Ebp fiber is involved in iron acquisition
252 in *E. faecalis* and might play some role in ferric ion reduction (17).

253
254 Electron conducting redox polymers, such as OsRP, are of large potential in
255 bioelectrochemical applications within environmental monitoring and energy
256 transformation. As demonstrated in this work Ndh3 and EetA are not important for
257 electron transfer from the quinol pool to OsRP (Fig. 3). OsRP apparently accepts
258 electrons directly from reduced DMK. Variants of redox hydrogel containing
259 covalently bound quinone can substitute for the lack of DMK in *E. faecalis* cells and
260 mediate glucose-dependent EET (20, 21). In conclusion, function of OsRP as
261 mediator in EET does not rely on components involved in EET mediated by ferric
262 ions. I.e., electron transfer mediated to an electrode by OsRP occurs via a route
263 distinct from that mediated with ferric ions. This aspect of material-cell interface is
264 directly applicable in the development and optimization of microbial electrochemical
265 systems to achieve efficient energy conversion designs.

266 MATERIALS AND METHODS

267 Growth of bacteria

268
269 Bacterial strains used in this work are listed in Table 1. *E. faecalis* strains were grown
270 in tryptic soy broth without dextrose (Difco) (TSB) with 1% (w/v) glucose added
271

272 (TSBG) and with or without hemin added, as before (18), or on 1.5 % (w/v) agar
273 plates containing TSBG, Todd-Hewitt (Difco), or Brain Heart Infusion (Difco) medium.

274

275 **Isolation of ferric reductase mutants**

276 An available collection of *E. faecalis* OG1RF EfaMarTn insertion mutants (18), stored
277 frozen as individual clones in 20% (w/v) glycerol in 96 well micro titer plates, was
278 replicated to Todd-Hewitt agar plates supplemented with 0.2 mM ammonium
279 ferrisulfate dodeca hydrate. The plates were incubated at 37 °C for one or two days
280 to obtain colonies. Then the agar plates were overlaid with soft agar (0.8% w/v agar)
281 containing 0.5 % glucose and 2 mM FerroZine™ (3-(2-pyridyl)-5,6-diphenyl-1,2,4-
282 triazine-4', 4''-disulfonic acid monosodium salt hydrate). Ferric reductase activity was
283 observed as pink coloration of colonies. Poorly staining colonies were immediately
284 picked and streaked on the same type of plates and the phenotype was confirmed
285 first by repetition of the ferric reductase activity staining of colonies and subsequently
286 by ferric reductase activity determination with washed cells grown in liquid medium.

287

288 **Identification of EfaMarTn insertions sites in chromosomal DNA**

289 The insertion site of the 2.1 kb transposon EfaMarTn (22) in the chromosome of *E.*
290 *faecalis* strain LFR9 and LFR16 was determined using inverse PCR after cleavage of
291 DNA with HindIII and exploiting primers invCATR2 and invGFPR1 for both DNA
292 amplification and sequence analysis, as described before (18). The transposon
293 insertion site in strains LFR1, LFR15 and LFR19 were determined by sequence
294 analysis of PCR products obtained using primers ndh3up and ndh3dw. The genotype
295 of strains ndh3::Tn and pplA::Tn were confirmed using primers ndh3up, ndh3dwn,
296 pplAup, pplAmid, invCATR2 and invGFPR1. Phusion DNA polymerase (Thermo
297 Scientific) was used for PCR. Sequence of primers are presented in Table S1.

298

299 **Ferric reductase activity assay of cells**

300 Cells were grown in 25 ml TSBG in a 250 ml Erlenmeyer flask at 200 rpm and 37 °C
301 to mid exponential growth phase OD₆₀₀ = 0.6 – 0.8 (90 – 180 min). After harvest of
302 the cells by centrifugation, 2 700 x g for 10 min at room temperature, and one wash
303 in 5 ml of TSB they were suspended in TSB to OD₆₀₀ = 1.2 and stored on ice and
304 analyzed the same day. Ferric reductase activity assayed using FerroZine™ was
305 determined at 37 °C in 0.92 ml in a 1ml cuvette containing TSB, 0.4% (w/v) glucose,

306 2 mM ammonium ferric sulfate, 1 mM FerroZine™, and cells of OD₆₀₀ 0.4. The assay
307 was started by the addition of 500 µl 2 mM ammonium ferric sulfate in TSB to the
308 cuvette containing the other ingredients. Formation of ferrous-Ferrozine™ complex
309 was monitored at 562 nm. Reductase activity was calculated based on the initial rate
310 of increase in absorbance, after subtraction of background value obtained in the
311 absence of Ferrozine™, and using the extinction coefficient 27.9 mM⁻¹ cm⁻¹ (19).

312
313 Activity with potassium ferricyanide as the substrate was determined at 37 °C in 1 ml
314 in a 1 ml cuvette containing 35 mM Tris/HCl pH 7.5, 0.4% (w/v) glucose, 1 mM
315 K₃Fe(CN)₆ and cells of OD₆₀₀ 0.4. Two cuvettes and a double beam
316 spectrophotometer was used. Ferricyanide (40 µl 25 mM) was added only to the
317 sample cuvette. This started the reaction which was monitored as reduction in
318 absorption at 420 nm over 2 min. Activity was calculated based on the extinction
319 coefficient 1.04 mM⁻¹ cm⁻¹.

320
321 Ferric reductase activities were determined using at least duplicate cell samples
322 grown independently and two or more assays were done per sample.

323 324 **Electrochemical measurements**

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

329 Electrochemical measurements were done at room temperature in phosphate buffer
330 (50 mM Na₂HPO₄/NaH₂PO₄, 100 mM KCl), pH 7.40 using a three-electrode system.
331 When indicated 0.5 mM potassium ferricyanide, added to the buffer, or osmium
332 complex modified redox polymer (OsRP) [Os(2,2'-bipyridine)₂-poly(N-
333 vinylimidazole)₁₀Cl]^{2+/+} (23) with a formal potential of 420 mV vs. the standard
334 hydrogen electrode (SHE), 10 mg mL⁻¹ in water, was applied as a mediators.
335 Spectrographic graphite rods (Alfa Aesar GmbH & Co., ø 3.05 mm) were prepared
336 and modified with *E. faecalis* cells and OsRP as described in (4) and used as
337 working electrodes.

338 Amperometric measurements were performed at a constant potential of 588 mV
339 versus the standard hydrogen electrode (SHE) controlled by a potentiostat (Zäta
340 Elektronik, Höör, Sweden) and using a wall jet electrochemical flow cell with a
341 connected working graphite, platinum counter and Ag/AgCl (0.1 M KCl) reference
342 electrode. All potentials in this work are given in reference to SHE. The electrolyte
343 solution was pumped through the system at a flow rate of 0.5 mL min⁻¹.

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

348 **Miscellaneous methods**

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

355

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364

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

Strain	EfaMarTn insertion position	Locus tag	Gene	Source of strain/reference
OG1RF	- ^a	n.a.	n.a.	(24)
LFR1	2 659 891	n.a.	intergenic (<i>ndh3-eetA</i>)	This work
LFR9	2 660 001	OG1RF_12511	<i>eetA</i>	This work
LFR15	2 658 400	OG1RF_12510	<i>ndh3</i>	This work
LFR16	2 658 088	OG1RF_12510	<i>ndh3</i>	This work
LFR19	2 659 648	OG1RF_12510	<i>ndh3</i>	This work
<i>ndh3::Tn</i>	2 658 965	OG1RF_12510	<i>ndh3</i>	(25) (strain RS12800)
<i>pplA::Tn</i>	2 657 173	OG1RF_12509	<i>pplA</i>	(25) (strain RS12795)
WY84	n.a. ^b	OG1RF_10330	<i>menB</i>	(26)

^a 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.

Strain	Relevant genotype	Hemin (8 μ M) added to the growth medium	Ammonium ferric sulfate reductase activity (%)	Ferricyanide reductase activity (%)
OG1RF	wild type	-	100 $\pm$ 15	100 $\pm$ 7
		+	10 $\pm$ 3	13 $\pm$ 7
WY84	<i>menB</i>	-	15 $\pm$ 4	13 $\pm$ 8
		+	11 $\pm$ 7	18 $\pm$ 6

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

Strain	Gene inactivated	Hemin added to the growth medium ^a	NADH oxidase activity (%) ^b
OG1RF		-	
		+	100 $\pm$ 6
ndh3::Tn	<i>ndh3</i>	-	< 8
		+	75 $\pm$ 11
pplA::Tn	<i>pplA</i>	-	< 8
		+	126 $\pm$ 6
WY84	<i>menB</i>	-	< 8
		+	< 8
LFR1	intergenic <i>ndh3</i> - <i>eetA</i>	-	46 $\pm$ 1
		+	< 8
LFR9	<i>eetA</i>	-	68 $\pm$ 2
		+	< 8
LFR16	<i>ndh3</i>	-	43 $\pm$ 2
		+	< 8

^a 2 μ M 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 ferricyanide. 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.