

Fe(III) reduction during pyruvate fermentation by *Desulfotomaculum reducens* strain MI-1

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ABSTRACT

Desulfotomaculum reducens MI-1 is a Gram-positive, sulfate-reducing bacterium also capable of reducing several metals, among which is Fe(III). Very limited knowledge is available on the potential mechanism(s) of metal reduction among Gram-positive bacteria, despite their preponderance in the microbial communities that inhabit some inhospitable environments (e.g., thermal or hyperthermal ecosystems, extreme pH or salinity environments, heavy metal or radionuclide contaminated sediments). Here, we show that in the presence of pyruvate, this micro-organism is capable of reducing both soluble Fe(III)-citrate and solid-phase hydrous ferric oxide, although growth is sustained by pyruvate fermentation rather than Fe(III) respiration. Despite the fact that Fe(III) reduction does not support direct energy conservation, *D. reducens* uses it as a complementary means of discarding excess reducing equivalent after H₂ accumulation in the culture headspace renders proton reduction unfavorable. Thus, Fe(III) reduction permits the oxidation of greater amounts of pyruvate than fermentation alone. Fe(III) reduction by *D. reducens* is mediated by a soluble electron carrier, most likely riboflavin. Additionally, an intracellular electron storage molecule acts as a capacitor and accumulates electrons during pyruvate oxidation for slow release to Fe(III). The reductase responsible for the transfer of electrons from the capacitor to the soluble carrier has not been identified, but data presented here argue against the involvement of *c*-type cytochromes.

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INTRODUCTION

Since the first evidence of microbial dissimilatory iron reduction emerged more than two decades ago (Lovley & Phillips, 1988; Myers & Nealson, 1988), awareness of the importance of this process has grown. It is now recognized as the main driving force for iron reduction in most non-sulfidogenic environments – with direct implications in the formation, dissolution, and weathering of minerals – as well as for organic matter oxidation in anoxic environments (Lovley, 1991; Canfield *et al.*, 1993; Fredrickson *et al.*, 1998; Frankel & Bazylinski, 2003).

The capability of iron-reducing bacteria (IRB) to transfer electrons to solid-phase Fe(III) – the most relevant form of Fe(III) in neutral pH environments – is striking because most respiratory processes rely on a soluble terminal electron acceptor (TEA) which diffuses into the cell and is reduced in the cytoplasm or periplasm, allowing for a ‘cell-contained’ electron transport chain. In the case of

solid-phase TEAs, bacterial cells require the electron transfer chain to include an extracellular step, the mechanism of which has been the subject of extensive research, focused primarily on two organisms, *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1 (Weber *et al.*, 2006; Ehrlich & Newman, 2009; and references within). For these two organisms, electron transfer models that share some general similarities have been described. Electron transport is mediated primarily by several multiheme *c*-type cytochromes, which sequentially transfer the electrons from the quinone pool in the cytoplasmic membrane, across the periplasm, to the outer membrane (Schroder *et al.*, 2003; Shi *et al.*, 2007). In order to transfer electrons from the outer membrane *c*-type cytochromes to extracellular TEAs, three major strategies have been identified. The first, reportedly used by both *S. oneidensis* and *G. sulfurreducens*, is direct contact of an outward-facing outer membrane *c*-type cytochrome with the solid-phase TEA (Lower *et al.*, 2001; Gralnick & Newman, 2007; Shi *et al.*,

2009; Inoue *et al.*, 2011). The second possibility, which also appears to be used by both model organisms, is the contact between the cell and the TEA through electron conductive pili (Reguera *et al.*, 2005; Gorby *et al.*, 2006). The final strategy, which is adopted by *S. oneidensis* but not by *G. sulfurreducens*, is the use of a soluble redox active compound, such as a flavin, capable of shuttling electrons from the final reductase in the cell to the TEA localized extracellularly (von Canstein *et al.*, 2008; Marsili *et al.*, 2008).

The thorough research that has been devoted to iron reduction by *G. sulfurreducens* and *S. oneidensis*, both Proteobacteria, is in contrast to the dearth of investigations of iron reduction by other micro-organisms, despite the wide phylogenetic variety of IRB that span across most bacterial phyla (Weber *et al.*, 2006). In particular, very little information is available concerning Gram-positive IRB, although they include at least 16 unique genera (Wrighton *et al.*, 2011a). Understanding iron reduction by Gram-positive bacteria is of particular interest in part because of their abundance in some environments (Burkhardt *et al.*, 2011; Sharp *et al.*, 2011) where Gram-negative IRB are not detectable. These are typically harsh environments where salinity (Emmerich *et al.*, 2012) or temperature is high (Nepomnyashaya *et al.*, 2010) or toxic metals (Burkhardt *et al.*, 2011) or other contaminants (Lin *et al.*, 2007) are present. Furthermore, to carry out extracellular electron transfer, Gram-positive IRB must face the challenge of having, as the outermost cell layer, a thick cell wall (up to 80 nm), and in some cases a glycoprotein surface layer (S-layer). To date, the proposed models for extracellular electron transfer in Gram-positive bacteria remain in their infancy. The secretion of an endogenous electron shuttle is a potential strategy that, as yet, has no experimental support; it has, however, been shown that certain bacteria (i.e., *Listeria monocytogenes*, *Thermoanaerobacter* sp., *Thermincola ferriacetica*, and *Thermincola potens*) require direct contact with the TEA, although it is unclear how electrons might be transferred across the cell wall (Deneer *et al.*, 1995; Marshall & May, 2009; Wrighton *et al.*, 2011b). Carlson *et al.* (2012) suggest that, in *T. potens*, the mechanism may be similar to the direct contact between cell surface and solid-phase TEA reported for the Gram-negative model bacteria, because evidence was found for the presence of surface multiheme cytochromes.

In this work, we investigated the reduction in soluble and solid-phase Fe(III) by the Gram-positive, sulfate-reducing bacterium *Desulfotomaculum reducens* MI-1 (isolated by Tebo & Obraztsova, 1998) with pyruvate as the electron donor. We aimed to identify the electron transfer mechanism, to ascertain whether *c*-type cytochromes are involved, whether reduction of solid-phase Fe(III) requires direct contact, and whether the micro-organisms gain any benefit from the reduction in Fe(III).

Throughout this study, we use TEA interchangeably with Fe(III) without necessarily implying a respiratory process.

MATERIALS AND METHODS

All manipulations were performed using standard anaerobic techniques. Media and stock solutions were boiled and purged with N₂ prior to autoclaving or filter sterilizing in serum bottles sealed with blue butyl rubber stoppers and aluminum crimp seals. Unless otherwise specified, all experiments were conducted in triplicates, and error bars in the plots represent the standard deviation values.

Micro-organism and growth conditions

Desulfotomaculum reducens strain MI-1 was grown anaerobically in basal Widdel low phosphate (WLP) medium amended with trace elements and vitamins (Bernier-Latmani *et al.*, 2010) at pH 7.1 ± 0.1. WLP medium amended with 0.05% yeast extract (Becton, Dickinson & Company, Sparks, MD, USA), 30 mM NaHCO₃ (Acros, Geel, Belgium), and 15 mM pyruvate (pyruvic acid 98%; Acros) was used for fermentative growth. Cells grown at 37 °C under these conditions were harvested in late exponential phase by centrifugation at 8000 × *g* for 10 min (Avanti centrifuge with JLA 91000 or JA-12 rotors, Beckman Coulter, Pasadena, CA, USA), washed in WLP basal medium, and used as an inoculum (10%) for Fe(III) reduction experiments.

For soluble or solid-phase Fe(III) reduction experiments, fermentative WLP medium was amended with 10 mM Fe(III)-citrate (Sigma, St. Louis, MO, USA) or hydrous ferric oxide (HFO) [prepared according to Lovley & Phillips (1986)]. Cultures were incubated at 37 °C and sampled for the concentrations of Fe(II), pyruvate, acetate, H₂, as well as for cell growth in an anaerobic chamber (Coy, Grass Lake, MI, USA) with an atmosphere of 2.5–3.5% hydrogen (balance nitrogen) with disposable syringes. HFO reduction experiments were also conducted in the presence of 1 μM and 10 μM riboflavin (Sigma) and sampled for Fe(II).

HFO-embedded in glass reduction assays

In order to test the requirement for direct contact between cells and HFO for reduction, HFO-embedded glass particles, henceforth referred to as glass-HFO, were prepared according to a slightly modified protocol from Lies *et al.* (2005). A volume (500 mL) of a 50 mM sterile FeCl₃ (Sigma-Aldrich) solution was added to 35 g of porous glass particles (Native CPG502 pore glass from Prime synthesis Inc., Aston, PA, USA: grain size, 75–125 μm; pore size, 50 nm; pore volume, 1.4 mL g⁻¹; specific surface area, 106 m² g⁻¹; bulk density, 0.29 g mL⁻¹). Fe(III) was

precipitated to HFO by the addition of NaOH (Lovley & Phillips, 1986) on the inner and outer surfaces of the particles. Excess HFO not precipitated with the glass particles was collected for use as a positive control for reduction. The glass particles were washed 10 times in 50 mM NaCl and five times in water to remove most of the outer surface HFO and dissolved NaCl from the glass particles. Washed glass-HFO particles were recovered by filtration (Whatman filter paper 587; pore size, 4–7 μm) and were air-dried prior to storage at room temperature. Average glass-HFO Fe content was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, ICPE-9000; Shimadzu Corporation, Kyoto, Japan) of particles digested in 70% HNO_3 .

Reduction assays of glass-HFO by *D. reducens* were carried out in WLP medium amended with ~4 mM Fe(III), in the form of glass-HFO, 0.05% yeast extract, 10 mM NaHCO_3 , 10 mM pyruvate as electron donor. The medium was inoculated with 10% fermentatively grown, washed, *D. reducens* cells. Positive control reduction experiments with HFO suspensions in water were carried out. Samples for soluble and total extractable Fe(II) were collected over time.

Fe(III) or AQDS reduction in spent medium

The presence of a soluble electron carrier was also probed by preparing spent medium obtained by filter-sterilization (0.2 μm pore size Filtropur S/S filters; Sarstedt, Nümbrecht, Germany) from stationary growth phase fermentation or HFO-reducing cultures. Fermentation spent medium was amended with either 1 mM anthraquinone disulfonate (AQDS) or with 5 mM Fe(III)-citrate; HFO-reducing spent medium was amended with 1 mM AQDS. Abiotic reduction of AQDS or Fe(III) in the spent media was tested spectrophotometrically by measuring A_{326} (for AQDS) or using the ferrozine assay (see below), respectively. A control involving fresh medium was also included.

Analytical techniques

Growth was quantified by direct counting of DAPI-stained cells (Vectashield, Burlingame, CA, USA) using epifluorescence microscopy (Eclipse E800, Nikon, Egg, Switzerland), or by measuring the optical density at 600 nm (OD_{600}) (Biophotometer, Eppendorf, Hamburg, Germany).

Concentrations of pyruvate, acetate, formate, and butyrate were measured by ion chromatography (DX-3000; Dionex, Sunnyvale, CA, USA) with an IonPac AS11-HC column. Elution was carried out using a gradient of 0.5–30 mM KOH.

Flavins present in cell-free growth medium were quantified by fluorescence ($\lambda_{\text{ex}} = 445 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$) (Syn-

ergyMx spectrofluorometer, Biotek Instruments GmbH, Luzern, Switzerland).

High-performance liquid chromatography (HPLC) was used for flavin identification according to a slightly modified method from Capo-chichi *et al.* (2000). Samples were eluted through a ZORBAX Eclipse Plus C18 reverse-phase column (2.1 \times 50 mm, 1.8 μm) (Agilent Technologies, Basel, Switzerland) with an isocratic eluent [15% acetonitrile in solution A (10 mM KH_2PO_4 and 15 mM $(\text{CH}_3\text{COO}^-)_2\text{Mg}$ adjusted to pH 3.4 with H_3PO_4)] at a flow rate of 0.5 mL min^{-1} for 5 min. The HPLC system was connected with a spectrofluorometer (1260 FLD Spectra; Agilent Technologies), set at $\lambda_{\text{ex}} = 445 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$. Riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) standards were prepared in solution A. Filtered (0.2 μm) growth medium was diluted 1:1 in solution A prior to elution. UV-VIS absorbance profile of filtered spent medium spiked with 10 μM RF was measured with a spectrophotometer (UV-2501PC, UV-VIS Recording Spectrophotometer; Shimadzu, Reinach, Switzerland).

Fe(II) samples were stabilized in 2 M HCl and stored at room temperature until quantification: samples were diluted in ferrozine (1 g l^{-1} ferrozine in 50 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer (Acros, Geel, Belgium)), and their concentration determined by measuring the absorbance at 562 nm (A_{562}) (SynergyMx spectrofluorometer, Biotek Instruments GmbH). H_2 in the headspace of cultures was measured with a gas chromatograph (GC-450, Varian, Middelburg, The Netherlands) using a molecular sieve column (CP81071: 1.5 m*1/8" Ultimetalsieve 13 \times 80–100 mesh) and a thermal conductivity detector (TCD).

qRT-PCR

Reverse transcription- and quantitative reverse transcription-polymerase chain reaction (RT-PCR and qRT-PCR, respectively) were performed on total RNA extracted from *D. reducens* cultures under different conditions: fermentation with or without 10 mM Fe(III)-citrate from inoculation or amended with Fe(III)-citrate during the exponential phase. The cultures were harvested after 0.7–1 mM Fe(III) was reduced, or in mid-exponential phase in the case of fermentation in the absence of Fe(III), and lysozyme-digested prior to storage at -80°C . Subsequently, RNA was extracted using the RNeasy extraction kit (Qiagen, Hilden, Germany). The quantity and quality of the RNA extracted were evaluated with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand synthesis was carried out using ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) and random hexamers d(N)-6 (Microsynth, Balgach, Switzerland) as primers. The 16S rRNA, *wrfA*, and *wrfH*

genes were targeted by RT-PCR and qPCR. For each gene, primers specific to *D. reducens* were used: 16S rRNA, Dred_16S_F (5'-AAA ACG GAG GAA GGT GGG GA-3'), and Dred_16S_R (5'-CTC CTT GCG GTT AGC TCA CC-3'); *nrfA*, *nrfA*_F (5'-AGA GTT TTA CGA GCC CCG GA-3'), and *nrfA*_R (5'-AAT GCT GGC CTG CTG ATA CG -3'); *nrfH*, *nrfH*_F (5'-CAT TAT GGA TCC CTG GGT TG -3'), and *nrfH*_R (5'-GTC CTG ACC ACG GTC ATT CT-3'). The primers for the 16S rRNA and *nrfA* genes were designed with Primer BLAST, those for the *nrfH* gene were designed by Junier *et al.* (2010). Further details on the RNA extraction procedure and qRT-PCR are described in the Supporting Information.

SDS-PAGE

Proteins were extracted from *D. reducens* fermentation cultures with or without Fe(III)-citrate amended at mid-exponential growth phase. Cultures were collected at mid-exponential phase in the case of fermentation conditions, and once it was established that Fe(III) reduction was occurring (>0.5 mM Fe(III) reduced) under Fe(III)-reducing conditions. Samples were processed as described in the Supporting Information performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on homogenized cell lysates. Gels were stained for all proteins with Coomassie blue or for proteins with peroxidase activity (such as ϵ -type cytochromes) using the tetramethylbenzidine (TMBZ)-hydrogen peroxide method (Thomas *et al.*, 1976).

Transmission electron microscopy

Whole mounts of the product of HFO reduction, deposited on carbon-coated copper grids (Quantifoil Micro Tools GmbH, Jena, Germany), were imaged with a FEI CM300UT FEG-UT microscope. Selected area electron diffraction (SAED) of micrometer-scale areas and Fourier Transforms of high-resolution transmission electron microscopy (HR-TEM) images were used for phase identification with the Java Electron Microscopy Software (JEMS) (Stadelmann, 2012); the structural data of iron minerals were taken from the Inorganic Crystal Structure Database (ICSD, FIZ Karlsruhe, Germany and NIST, U.S. Department of Commerce, 2012).

Glass-HFO samples were prepared in the anoxic chamber by fixation with 2.5% glutaraldehyde (Grade I, 25%, Sigma-Aldrich) followed by dehydration in pure grade ethanol (Sigma-Aldrich) series (25%, 50%, 75%, 90%, 100% in water) and embedding for a week and at room temperature in LR-white resin (Electron Microscopy Science, Hatfield, PA, USA), which polymerized at 60 °C overnight. An Ultracut E ultramicrotome (Reichert-Jung) and an ultrasonic (oscillating) 35° knife from Diatome (Biel, Switzerland)

land) were used to cut resin sections, which were placed on carbon-coated copper grids. Local chemical analysis of epoxy-embedded glass-HFO thin sections (40–60 nm) was performed using X-ray energy-dispersive spectroscopy (EDS) in the scanning transmission electron microscopy (STEM) mode in a FEI Tecnai Osiris microscope (200 kV). EDS data interpretation was carried out with the ESPRIT software (Bruker, Billerica, MA, USA).

RESULTS AND DISCUSSION

Fe(III) reduction

With pyruvate as an electron donor, *D. reducens* is capable of reducing both soluble (i.e., Fe(III)-citrate) and solid-phase (i.e., HFO) Fe(III); killed, lysed, and no-cell controls did not reduce Fe(III), nor could citrate alone support soluble Fe(III) reduction (Fig. S1). Figure 1A and B shows increasing concentrations of Fe(II) over time, as well as pyruvate consumption, buildup of acetate, H₂ and small amounts of butyrate and formate from pyruvate oxidation, and an increase in cell counts during reduction of Fe(III)-citrate and of HFO, respectively. In the case of Fe(III)-citrate reduction, there is no evidence for the formation of a Fe(II) precipitate, while in the case of HFO – despite the lower rate and extent of the reduction – the solid phase shifted in color from orange–brown to black as the reduction proceeded, suggesting the accumulation of a Fe(II)-bearing solid phase. This was confirmed by SAED patterns and HR-TEM diffractograms, which identified the solid phase as magnetite (Fig. 2).

During the reduction of both soluble and solid-phase Fe(III), pyruvate – the electron donor – was completely consumed within a few days. At the same time, the acetate concentration in solution built up to reach a plateau, and cell growth reached stationary phase. Conversely, Fe(II) accumulation did not follow the same time scale and continued even after the depletion of the electron donor [after 25 days, 8 mM Fe(III) citrate and 4.4 mM HFO were reduced to Fe(II) (Fig. S2)], suggesting that pyruvate oxidation and TEA reduction are temporally uncoupled (Fig. 1). These observations are consistent with the utilization of pyruvate as a substrate for fermentative growth, rather than as an electron donor for Fe(III) respiration. If fermentation is the dominant metabolism, is there any benefit to the cells from Fe(III) reduction?

We address this question by considering pyruvate consumption by a fermentation culture, in the absence of Fe(III) (Fig. 1C). Comparing Fig. 1A,B to Fig. 1C, we found that in the absence of Fe(III), pyruvate is initially consumed at approximately the same rate as in the presence of Fe(III), but after 1 day of incubation, the consumption rate decreases, resulting in the persistence of a significant amount of pyruvate (~8 mM) even after

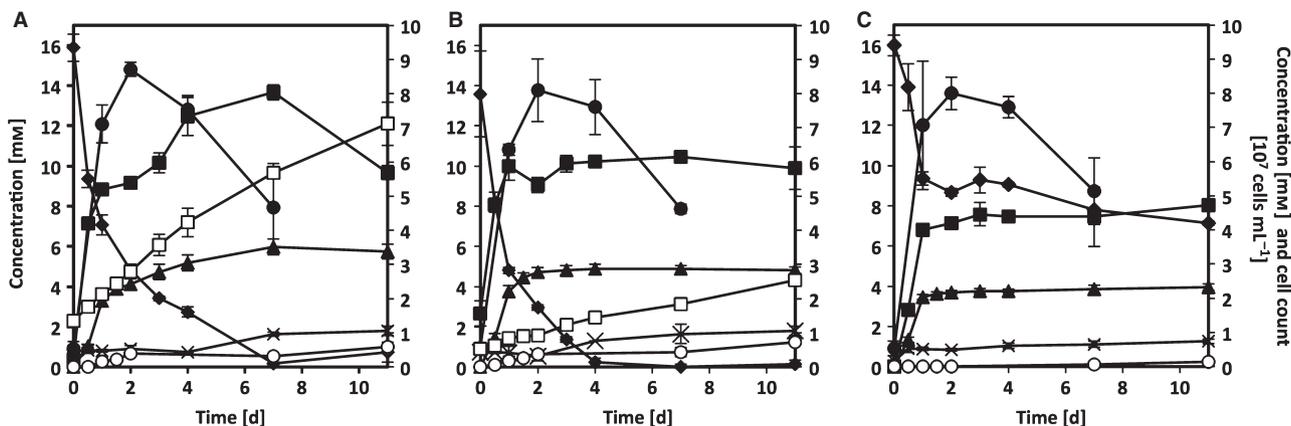


Fig. 1 Evolution of *D. reducens* cultures in the presence of Fe(III)-citrate (A) or HFO (B) or in the absence of Fe(III) (C): concentrations of H_2 (full triangles), pyruvate (full diamonds) and acetate (full squares) are to be read on the left axis; concentrations of Fe(II) (open squares), butyrate (open circles), formate (crosses) and cell count (full circles) are to be read on the right axis.

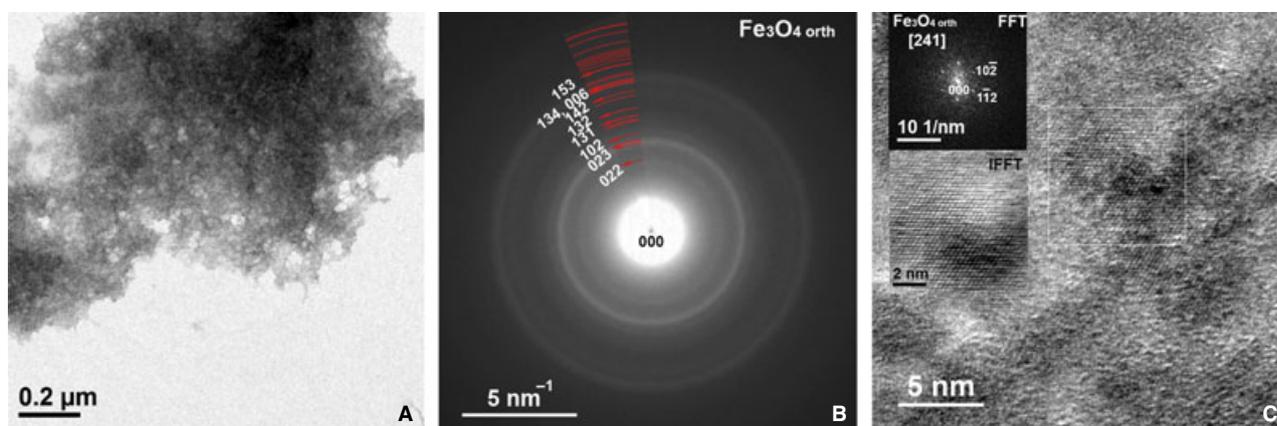


Fig. 2 TEM image (A), selected area electron diffraction (SAED) patterns with the strongest reflections indicated in red (B) and high resolution TEM (HR-TEM) image showing the product of HFO reduction to be orthorhombic magnetite (Fe_3O_4) (C). The insets in the HR-TEM image correspond to the HR-TEM diffractogram (Fourier transform, FFT) and the Fourier filtered image (IFFT) from the selected particle.

10 days. In contrast, Fe(III)-containing cultures deplete pyruvate completely after 4–7 days (Fig 1A,B). Consistent with the smaller amount of consumed pyruvate, lower concentrations of organic acids accumulate in the absence of Fe(III). In contrast, cell counts are comparable in the presence or absence of Fe(III). Because the decrease in the rate of pyruvate consumption in the Fe(III)-free culture temporally coincides with the H_2 concentration plateau, we surmised that the accumulation of significant ($\sim 4 \text{ mM}$, corresponding to a partial pressure (pH_2) of $\sim 10^4 \text{ Pa}$) H_2 in the headspace may inhibit further fermentation of pyruvate. Fermentation inhibition due to high pH_2 (in the order of 10^4 Pa , or less in some cases) has been previously reported for several micro-organisms: *Clostridium acetobutylicum* (Yerushalmi *et al.*, 1985), *Pyrococcus furiosus* (Schäfer & Schönheit, 1991), *Caldicellulosiruptor saccharolyticus* (van Niel *et al.*, 2003), and anaerobic consortia used for H_2 production (Valdez-Vazquez *et al.*, 2006). To support this hypothesis, we evaluated the effect of

removing H_2 by purging the headspace of a Fe(III)-free *D. reducens* fermentation culture after pyruvate consumption had stopped. Consistently with the hypothesis that H_2 accumulation in the serum bottle inhibited further pyruvate fermentation, we observed a renewed buildup of the gas and resumption of pyruvate consumption (Fig. 3).

Hence, our findings strongly indicate that when Fe(III) is present, it can be used as an electron sink alternative to protons, which allows the bacteria to divert electrons derived from pyruvate fermentation to the metal, thus overcoming the obstacle imposed by the strong backpressure of H_2 accumulated in the headspace. The role played by Fe(III) during pyruvate fermentation by *D. reducens* is consistent with reports of bacteria using metal reduction as a means of eliminating excess reducing equivalents derived from electron donor oxidation (Lovley, 2006).

The temporal decoupling of electron donor oxidation and TEA reduction observed not only supports the conclu-

sion that Fe(III) reduction by *D. reducens* is not a respiratory process, but it also suggests electron storage. In order for the electrons to be slowly released to Fe(III) even after electron donor depletion, they must be stored within a molecule acting as a capacitor. The concept that bacterial cells may oxidize an available electron donor, accumulating electrons for later release to an electron acceptor has been proposed previously for *G. sulfurreducens* and *S. oneidensis* (Esteve-Nunez *et al.*, 2008; Schuetz *et al.*, 2009; Bonanni *et al.*, 2012). In contrast to *Geobacter* sp. and *Shewanella* sp., however, *D. reducens* is unlikely to use *c*-type cytochromes as electron capacitors, as they are not upregulated during Fe(III) reduction (as described in detail below). The capacitor could be a compound accumulated extracellularly such as H₂ or formate. Other plausible candidates are intracellular molecules (e.g., storage polymers, ferredoxin, NADH, quinones). Very low concentrations of formate were detected in the reduction medium (Fig. 1A,B), and it was found not to be a suitable electron donor for Fe(III) reduction by *D. reducens* (Fig. S1b), excluding it from playing a significant part in Fe(III) reduction. H₂ accumulates significantly in the system; however, if it were the capacitor providing the electrons for Fe(III)-reduction, its concentration would be expected to decrease over time, which is not the case. Furthermore, we performed a separate experiment to test the effect of substituting the H₂-rich headspace of an HFO-reducing, pyruvate-fermenting culture with sterile N₂. We found that removing H₂ did not affect the reducing

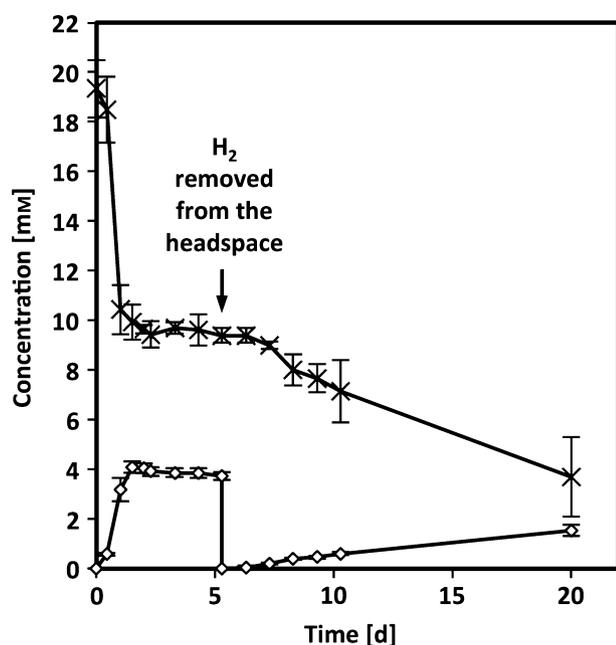


Fig. 3 Pyruvate (crosses) concentration in a serum bottle containing a fermenting culture (in the absence of Fe(III)) and effect of the replacement of all H₂ (diamonds) accumulated in the headspace by N₂ (at 5 days): pyruvate consumption resumes and fresh H₂ is released in the headspace.

activity of the culture, proving that electrons are not stored in H₂ (Fig. S3) and that electron transfer from the cells to the TEA does not include an intermediate H₂ step. Having excluded the most likely candidates for the role of capacitor, we propose that it must be an intracellular molecule. Microscopy observations show that during stationary phase, the cell morphology of *D. reducens* evolves from rod-shaped to round bodies (that are not spores) (Fig. S4), within which dark regions are visible. We hypothesize that these may be reduced carbon species, by analogy to other several sulfate-reducing bacteria, for example, *Desulfovibrio* spp. (Fareira *et al.*, 1997), *Desulfobulbus propionicus* (Stams *et al.*, 1983), *Desulfonema magnum* (Hai *et al.*, 2004), that are known to accumulate storage polymers (e.g., polyglucose (PG), polyhydroxybutyrate (PHB), glycogen) in the presence of excess energy or carbon source, when other growth conditions are restrictive (Preiss, 1984; Fareira *et al.*, 1997). PG was also shown to serve as a source of electrons for sulfate reduction in *Desulfovibrio* and *Desulfobulbus* spp. (Stams *et al.*, 1983). Figure S5 shows that, indeed, the round bodies store electrons and are capable of transferring them to Fe(III)-citrate.

During pyruvate fermentation, about 8.1 mM of electrons are unaccounted for if one considers the consumption of pyruvate and the production of acetate, butyrate, formate, H₂, and biomass. In the presence of Fe(III)-citrate and of HFO, about 8.5 and 9.2 mM of electrons are unaccounted for, respectively. Carbon balance calculations, presented in Table 1, indicate that there is excess carbon in all cases. However, only in one instance is there sufficient excess carbon to store all the unassigned electrons. Indeed, in the case of Fe(III)-citrate – containing the highest carbon excess –, there is 15.5 mM of C that could represent a storage compound. If we consider PHB as a possible compound, it can store 2e⁻ per molecule – 7.75 mM e⁻ in total, in our system – and thus comes close to representing a sink for all the unassigned electrons (corresponding to 8.5 mM). Thus, it is possible that reduced polymers (such as polyhydroxybutyrate, PHB) could be synthesized and could serve as a source of electrons for Fe(III) reduction. However, in the case of HFO and fermentation alone, there must be another (or several other) electron storage molecule(s). Potential candidates are ferredoxin, quinones, and NAD (although the latter is less likely to be involved as it plays no role in the pathway of pyruvate fermentation – see pathway in Fig. S6). For most of these compounds, iron reduction [E₀'(Fe³⁺/Fe²⁺) ~ 0–0.4 V, depending on the species: E₀'(Fe(III)-citrate/Fe²⁺) ~ 0.37 V, E₀'(Ferrihydrite/Fe²⁺) ~ 0 V (Thamdrup, 2000), E₀'(Fe(OH)₃/Fe²⁺) = 0.118 V (Dolfig *et al.*, 2007)] is thermodynamically favorable [E₀'(Fd_{ox}/Fd_{red}) = -0.398 V, E₀'(Ubiquinone/Ubiquinol) = 0.113 V, E₀'(Menaquinone_{ox}/Menaquinone_{red}) = -0.074 V, E₀'(NAD/NADH) = -0.320 V (Thauer *et al.*, 1977)].

Table 1 Comparison of carbon and electron balances during pyruvate fermentation in the absence and presence of soluble or solid-phase Fe(III). This table shows that there is an abundance of electrons that are unaccounted for in all cases. Based on the C balance, only in the case of Fe(III) citrate reduction, there is sufficient excess carbon that could serve as a sink for these electrons

	Fermentation			Fermentation + Fe(III) citrate			Fermentation + HFO		
	Amount consumed or formed (mM)	e ⁻ donated/accepted (mM)	Carbon content (mM)	Amount consumed or formed (mM)	e ⁻ donated/accepted (mM)	Carbon content (mM)	Amount consumed or formed (mM)	e ⁻ donated/accepted (mM)	Carbon content (mM)
Pyruvate	8.8	17.7	26.5	15.2	30.3	45.5	13.4	26.8	40.2
Acetate	8.0		16.0	9.7		19.3	11.2		22.5
H ₂	4.0	7.9		5.7	11.5		4.8	9.6	
Butyrate	0.1	0.6	0.6	0.6	2.4	2.4	0.7	2.9	2.9
Formate	0.6	1.1	0.6	0.6	1.2	0.6	0.6	1.2	0.6
CO ₂	7.4		7.4	6.0		6.0	6.5		6.5
Fe(II)	–			6.7	6.7		3.9	3.9	
Biomass*			1.60			1.74			1.62
Balance		8.1	0.40		8.5	15.5		9.2	2.8

*This value was estimated from cell count, and a C cell content of 2.4e-13 g per cell [intermediate value among the wide range reported in literature (Fagerbakke *et al.* (1996) and Fukuda *et al.* (1998)].

D. *reducens* can reduce HFO without direct contact

With the goal of testing the requirement for direct contact with the TEA, we conducted glass-HFO reduction experiments. Figure 4 shows that glass-HFO can be reduced by the cells at the same rate as directly accessible HFO. An EDS map of a glass-HFO thin section prior to and after 20 days of incubation is shown in Fig. 5E. Quantitative analysis of the map determined the concentration of Fe inside the beads, relative to SiO₂, to be ~0.2 and 0.1 atomic mass % before and after incubation, respectively. This is consistent with the fact that a significant fraction of the reduced HFO in the glass particles was soluble (1.4 mM of 2 mM) and thus lost from the solid phase. SEM observations of glass-HFO and elemental microanalysis (Fig. S7) prior to reduction experiments showed that

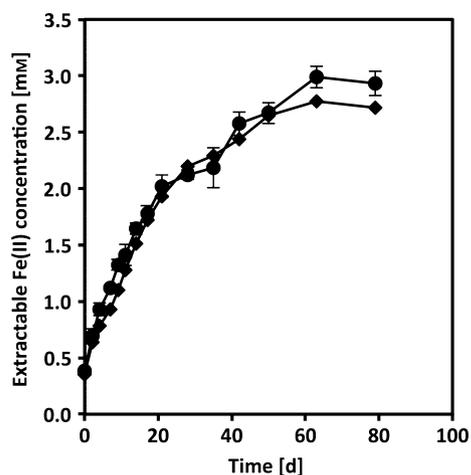


Fig. 4 Fe(II) build up over time as a result of glass-HFO (diamonds) and suspended HFO (circles) reduction by *D. reducens*.

small amounts of Fe(III) not removed from the washing treatments are present on the outer surface of the particles. However, surface Fe(III) is unlikely to account for the total content measured by ICP-OES: 7.2 mg Fe g⁻¹ particle. This is confirmed by STEM observations and EDS mapping of thin sections of these particles showing iron to be also distributed within the ~50 nm pores (Fig. 5A,B and C), thus inaccessible to cells by direct physical contact. Therefore, the ability to reduce glass-HFO suggests that Fe(III) – in particular HFO – reduction by *D. reducens* with pyruvate as electron donor entails mediation by a soluble electron carrier.

A reduced soluble compound is present in the spent medium of cultures fermenting pyruvate

AQDS was used as a TEA to test for the presence of soluble reduced compounds in spent HFO reduction medium. A sample from the HFO-growing culture was filter-sterilized at inoculation time (i.e., prior to the onset of HFO reduction), and another sample collected and filter-sterilized while HFO reduction was underway. Both samples were amended with 1 mM AQDS. After 48 h, measurement of residual AQDS revealed that 40% of the AQDS had been reduced to AH₂DS in the medium collected when HFO reduction was ongoing, while none was reduced in the medium collected before cells started growing and reducing HFO. This result suggests that a reduced compound, potentially involved in Fe(III) reduction, is released into the medium by the cells during HFO reduction with pyruvate as an electron donor.

Filter-sterilized spent medium from a culture grown fermentatively in the absence of Fe(III) was also amended with AQDS. Consistently with the HFO spent medium,

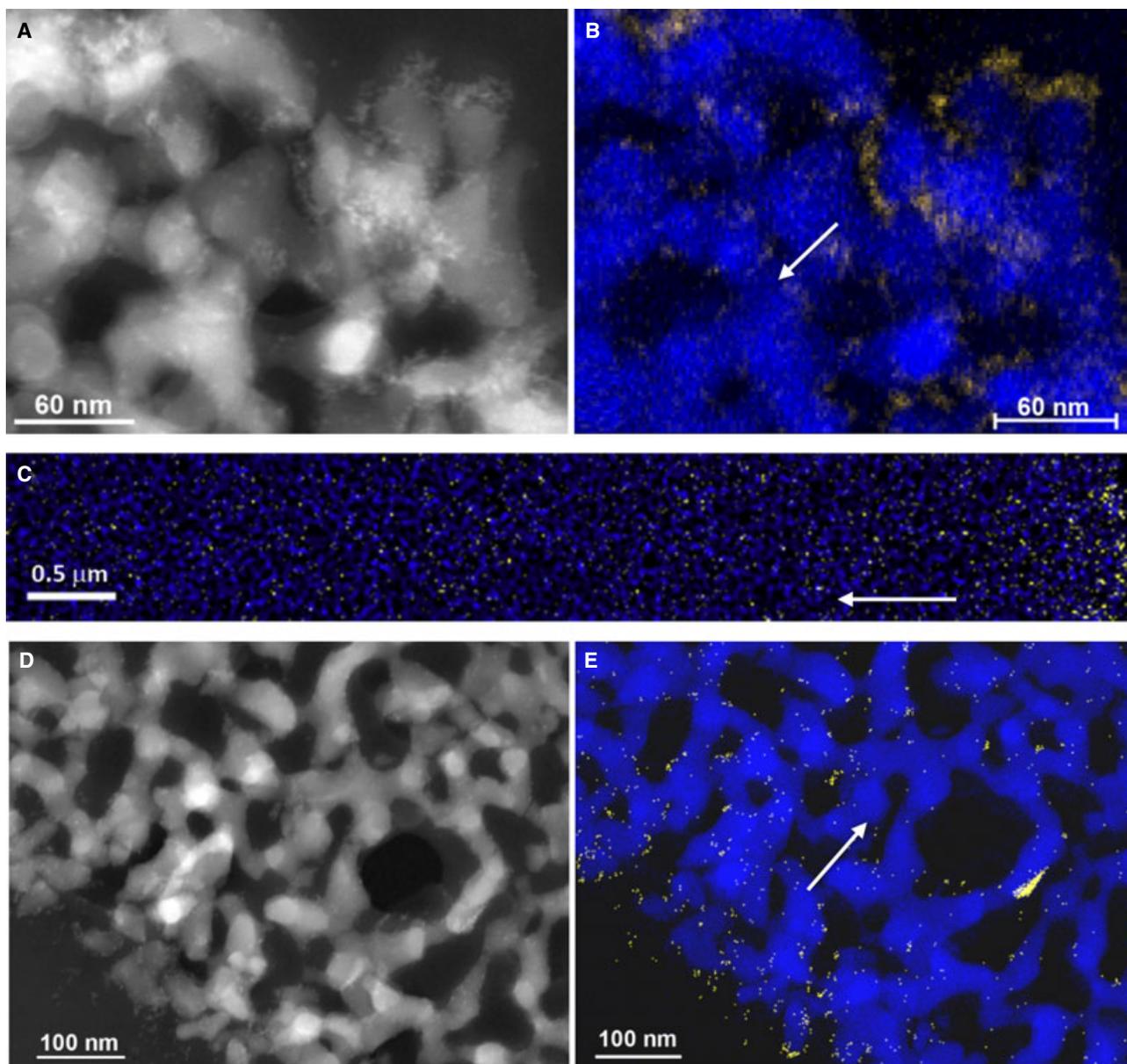


Fig. 5 STEM images (A and D) and EDS map of Si and Fe (B,C and E) of glass-HFO particles incubated with (D and E) and without (A,B and C) cells. In each map the direction from the surface towards the inside of the glass particle is indicated by an arrow. The fraction of Fe relative to SiO_2 obtained from EDS quantitative analysis is 0.2% for (B) and (C), and 0.1% for (E). Yellow represents Fe and blue Si.

the fermentation medium reduced AQDS by about 30% in 3 days, with a total of 0.54 mM electrons transferred (0.27 mM of AQDS was reduced), indicating that the reduced soluble compound is released during pyruvate fermentation and not only in the presence of HFO.

Additionally, we aimed at probing whether the soluble compound is secreted in sufficient amount during pyruvate oxidation to account for all the Fe(III) reduction observed or whether it is replenished by the cells. To probe this question, we used cell-free fermentation spent medium and found that Fe(III) was reduced to a much lesser extent

than AQDS: only 0.17 mM electrons were transferred without further reduction even after a month (Fig. S8). The limited efficacy of the fermentation spent medium, and presumably of the soluble compound, to reduce Fe(III) as compared to AQDS (Fig. S8) suggests that the electron shuttle is limited in quantity. Replenishment of the soluble compound by the cells could be achieved via redox cycling of the carrier or continuous release of its reduced form.

We probed the potential role of SO_4^{2-} as an electron shuttle, because *D. reducens* is a sulfate-reducing bacte-

rium, and trace amounts of this anion are present in the TE and YE. Sulfide produced by sulfate reduction could abiotically reduce Fe(III) and cycle back through sulfate reduction. If this were true, catalytic amounts of sulfate would be responsible for Fe(III) reduction. Because of the fastidiousness of MI-1 (see Fig. S9), we could not directly use WLP depleted of trace elements and yeast extract (likely to contain some sulfate), so instead we used, as an

Fe(III)-reduction medium, cell-free spent fermentation medium stripped of S^{2-} by precipitation as FeS. Fe(III)-citrate reduction tests by *D. reducens* were carried out in unmodified spent fermentation medium, spent fermentation medium stripped of S^{2-} , and fresh WLP medium (Fig. S10). The reduction curves in the three cases overlap almost perfectly, indicating that removing the sulfide from the system does not impact Fe(III) reduction and thus that

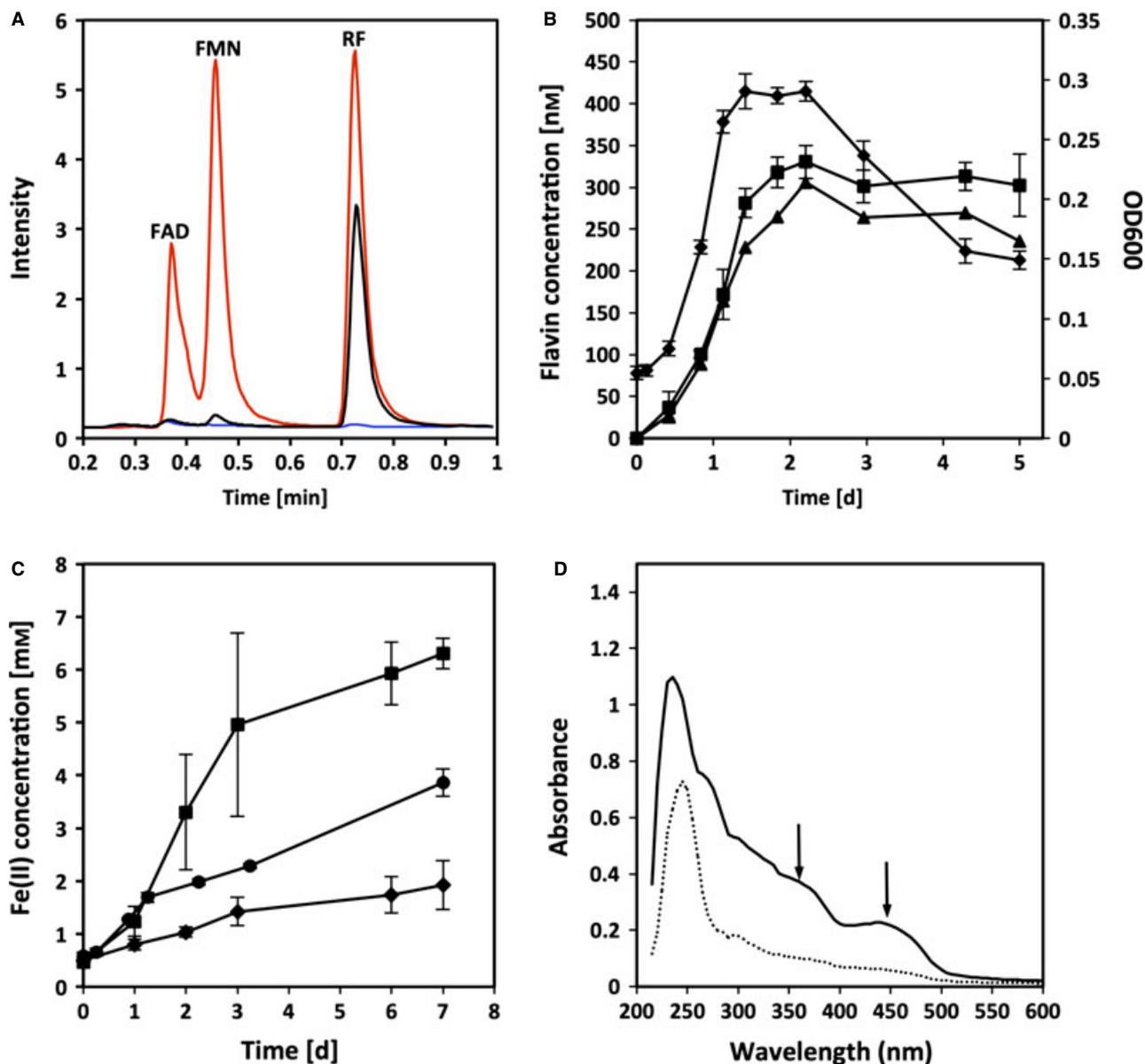


Fig. 6 (A) HPLC chromatograms of: a mix of FAD, FMN and RF standards (red curve), fresh WLP medium (blue curve), and filtered growth medium at 40 h of incubation (black curve). (B) Concentration of total flavins over time during fermentation in the presence (triangles) and absence (squares) of HFO, and growth curve for the culture without Fe(III) (diamonds). (C) Fe(II) accumulation due to HFO reduction by pyruvate fermentation cultures amended with 0 μM (diamonds), 1 μM (circles), and 10 μM (squares) of RF. (D) UV-VIS spectra of filtered spent growth medium from an HFO reducing culture spiked with 10 μM RF, measured anoxically (dashed curve) and after oxidation of the same sample (bold curve); the two arrows indicate the positions of the characteristic peaks of oxidized flavins (Macheroux, 1999). The appearance of diffuse peaks after oxidation clearly indicates the presence of flavins in the sample, present in their reduced form prior to oxidation.

the small amount of SO_4^{2-} present in the complete WLP medium does not play a significant role in Fe(III)-reduction by *D. reducens*.

As flavins have been reported to act as the electron shuttles for Fe(III) reduction carried out by *S. oneidensis* (von Canstein *et al.*, 2008; Marsili *et al.*, 2008), we performed a bulk spectrofluorometric measurement of cell-free spent fermentation medium to test for their presence. We found that after 24 h of growth, they are present at a concentration of approximately 170 nM. In order to verify that the fluorescing compound was indeed a flavin, and to identify its composition (whether RF, FMN or FAD), we analyzed the sample by HPLC. We found that a small FAD peak is present in the background (fresh WLP medium) but does not increase with cell growth. Conversely, FMN and RF, which are absent in fresh WLP medium, build up in the medium during cell growth (Fig. 6A), with RF representing 93% of the secreted flavin. To verify that flavins are present in the spent growth medium as a result of active release into the medium rather than cell lysis, we monitored their concentration over time by spectrofluorometry. We found that the flavin concentration curve follows the growth curve and that flavins are secreted by *D. reducens* during pyruvate fermentation regardless of the presence of Fe(III) in the system (Fig. 6B). Interestingly, the maximum concentration of flavins (i.e., 350 nM) measured in solution was found to be comparable with the amounts of extracellular flavins secreted by *S. oneidensis* MR-1 (i.e., 700 nM according to von Canstein *et al.*, 2008, 250–500 nM according to Marsili *et al.*, 2008) for solid-phase Fe(III) reduction.

Based on these observations, we suggest that flavins released from the cells as metabolites of pyruvate fermentation could act as electron carriers for Fe(III) reduction in *D. reducens*. To further validate this hypothesis, we compared the HFO reduction rate in the absence and in the presence of added RF (1 and 10 μM) and found that, indeed, spiking the culture with additional RF increases the rate of reduction (Fig. 6C). In addition, the UV–VIS spectrum of spent WLP medium during HFO reduction in the presence of 10 μM RF shows that the RF is reduced (Fig. 6D), proving that *D. reducens* is capable of reducing this compound.

Overall, we show, for the first time, that a Gram-positive bacterium is able to carry out solid-phase electron acceptor reduction through the intermediate of an endogenous soluble electron carrier, namely RF (and small amounts of FMN). Oxidized endogenous electron carrier is replenished by the cells by re-reduction and, during growth, *de-novo* secretion. Previous reports of solid-phase reduction by Gram-positive bacteria involving electron carriers only included exogenous molecules such as AQDS or an exudate from a Gram-negative bacterium (Milliken & May, 2007; Pham *et al.*, 2008).

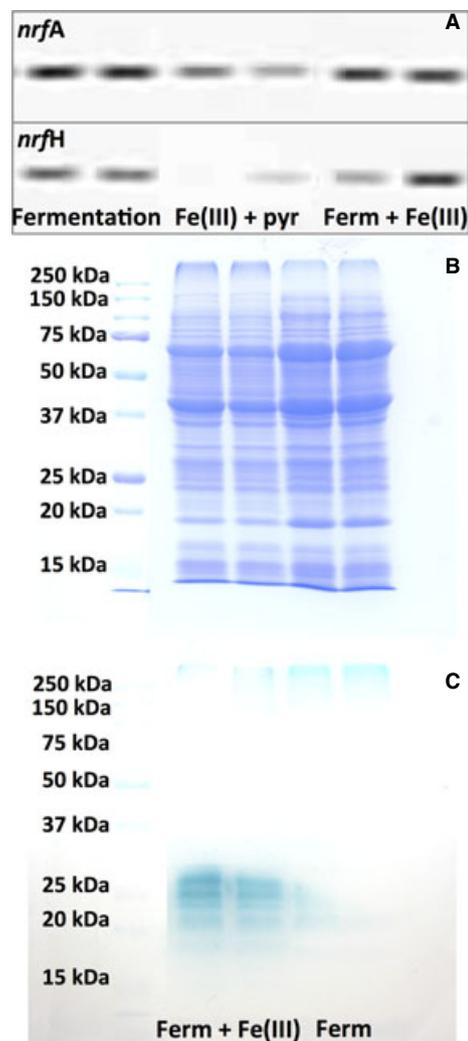


Fig. 7 Expression of the two *c*-type cytochromes encoded in the genome of *D. reducens*, NrfA and NrfH. (A) Electrophoresis gel showing the product of RT-PCR for *nrfA* and *nrfH*: from left to right in duplicate lanes: pyruvate fermentation, Fe(III)-citrate reduction, fermenting culture amended with iron at mid exponential growth phase. (B) and (C) SDS-PAGE gels stained with Coomassie-blue or TMBZ, respectively, of total protein extract from cultures under two different conditions; the tested conditions, each in duplicate lanes, are: pyruvate fermentation amended with Fe(III)-citrate at mid exponential growth phase and pyruvate fermentation. The bands reacting with the heme-stain in the fermentation amended with Fe(III) sample do not correspond to the predicted sizes for NrfA or NrfH, 48 kDa and 17 kDa, respectively.

Investigation of the involvement of *c*-type cytochromes in Fe(III) reduction by *D. reducens*

The necessary involvement of cells for Fe(III)-reduction to proceed implies an enzymatic step that allows the transfer of electrons from the capacitor to the carrier and potentially directly to soluble Fe(III)-citrate. In all the Gram-negative bacteria whose mechanism of Fe(III) reduction has been investigated and described, including the cases that involve electron shuttles and capacitors (von Canstein *et al.*, 2008;

Table 2 qRT-PCR results: comparative expression of *nrfA* and *nrfH* relative to 16S rRNA under different conditions ($2^{-\Delta C_t}$) and relative to fermentation ($2^{-\Delta\Delta C_t}$)

Conditions	<i>nrfA</i>		<i>nrfH</i>	
	$2^{-\Delta C_t}$	$2^{-\Delta\Delta C_t}$	$2^{-\Delta C_t}$	$2^{-\Delta\Delta C_t}$
Fermentation	1.2E-04	1.0E+00	2.4E-05	1.0E+00
Fermentation + Fe(III)	9.0E-05	7.6E-01	1.6E-05	6.8E-01
Fe(III)-citrate + pyruvate	2.8E-06	2.4E-02	5.4E-07	2.3E-02

Esteve-Nunez *et al.*, 2008; Marsili *et al.*, 2008; Schuetz *et al.*, 2009; Bonanni *et al.*, 2012), multiheme *c*-type cytochromes play a seminal role in the process (Shi *et al.*, 2007). Their key role was, in most cases, proven by deletion mutagenesis (Myers & Myers, 1997; Leang *et al.*, 2003; Lloyd *et al.*, 2003; Mehta *et al.*, 2005; Bucking *et al.*, 2010; Coursolle & Gralnick, 2012). We performed gene expression and proteomic experiments to probe the involvement of *c*-type cytochromes in Fe(III) reduction by *D. reducens*, although the lack of genetic system for this micro-organism did not allow genetic confirmation. Our targets were the only two genes present in the genome of strain MI-1 that encode for proteins containing binding domains for heme *c* (CXXCH): *nrfH* and *nrfA* (Junier *et al.*, 2010). NrfH and NrfA are predicted to be tetraheme cytochromes. RT-PCR carried out with *D. reducens*-specific primers for *nrfH* and *nrfA* and showed that the target genes appear to be downregulated during Fe(III)-citrate reduction as compared to fermentation (Fig. 7A). A very weak band is visible for one of the Fe(III)-citrate reduction duplicate samples. Weak bands are also detectable for *nrfA* in the case of Fe(III) reduction. In contrast, the bands for both genes are significantly more pronounced under fermentation conditions, although the *nrfH* band appears slightly weaker. qRT-PCR was performed to confirm these results quantitatively (Table 2). The results obtained with this method were consistent with the visual results of the RT-PCR electrophoresis gel: the expression level of *nrfA* and *nrfH* relative to 16S rRNA ($2^{-\Delta C_t}$) is approximately two orders of magnitude lower during Fe(III) reduction than during fermentation.

With the goal of further validating these expression data, which point to the lack of involvement of *c*-type cytochromes in Fe(III) reduction by *D. reducens* with pyruvate as an electron donor, we conducted SDS-PAGE with total protein extracts from cells fermenting pyruvate or reducing Fe(III)-citrate, which was added to a mid-exponential fermentation culture. Several bands reacting to the peroxidase-specific stain are visible for the fermentation culture amended with Fe(III) (Fig. 7C); however, the positions of these bands (~27–20 kDa) do not correspond to the predicted sizes for either NrfA or NrfH, 48 and 17 kDa, respectively. The lane corresponding to the fermentation sample exhibits weak staining at the upper edge of the gel,

suggesting that a peroxidase-type protein may not have migrated very far into the gel. Because NrfA and NrfH are predicted to form a transmembrane complex, based on the presence of transmembrane helices in the amino acid sequence, we considered it possible that the peroxidase-staining and non-migrating band in the fermentation sample lane could be a membrane complex containing NrfA or NrfH, or both. Therefore, we excised this band, as well as the peroxidase-reacting bands in the fermentation amended with Fe(III) sample, and analyzed them by LC-MS/MS to search for peptides forming significant coverage of the sequence of NrfH or NrfA (Table S1). We were unable to find any peptides belonging to *c*-type cytochromes or to identify the protein(s) with known peroxidase activity in any of the bands.

We conclude that, in contrast to Gram-negative IRB, there is no evidence for the involvement of *c*-type cytochromes in Fe(III) reduction by *D. reducens*. Interestingly, no *c*-type cytochrome nor orthologs of *nrfA* or *nrfH* were identified in the draft genome sequence of *Desulfotomaculum hydrothermale* Lam5^T, also reported to be capable of reducing Fe(III) with pyruvate as the electron donor (Haouari *et al.*, 2008; Amin *et al.*, 2013).

SUMMARY

In this work, we showed that *D. reducens* MI-1, a Gram-positive, sulfate-reducing bacterium, is capable of reducing

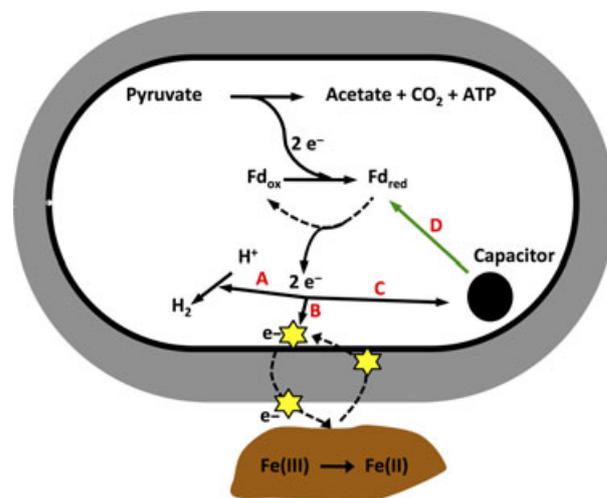


Fig. 8 Schematic of the proposed mechanism of Fe(III) reduction during pyruvate fermentation. During fermentation in the absence of Fe(III), the reduced ferredoxin releases electrons onto protons (pathway A), reducing them to H₂ until this reaction becomes unfavorable. At this point, some electrons can be stored in polymeric storages (e.g., PHB) or in other redox active molecules (e.g., ferredoxin, NAD, quinones) (pathway C). When Fe(III) is present, the reduced Fd can transfer electrons to a soluble electron carrier, likely to be flavins (pathway B), which in turn reduces Fe(III). Also, under these conditions the electron capacitor can return electrons to the Fd (pathway D), allowing for additional Fe(III) reduction.

both soluble and insoluble Fe(III) during pyruvate fermentation. Despite the fact that this micro-organism does not respire iron under these conditions, and thus does not directly conserve energy from the reduction, it does gain some benefit from the presence of Fe(III): the transfer of electrons to Fe(III) allows the bacterium to proceed with pyruvate oxidation even if the accumulation of H₂ precludes further reduction in the traditional electron sink, protons. The reduction of Fe(III) involves the storage of electrons, allowing Fe(III) reduction even after the depletion of the electron donor. Electrons are transferred from this capacitor to a soluble carrier (RF and FMN), which is released into the growth medium and, in turn, reduces Fe(III). It is unclear how the reduction of the carrier by the electron capacitor ensues and what enzyme(s) mediate(s) this reaction. However, we have shown that the involvement of *c*-type cytochromes is unlikely. A schematic of the reduction mechanism that we propose is depicted in Fig. 8. This is the first time a Gram-positive bacterium has been shown to carry out solid-phase reduction using an endogenous soluble electron carrier.

These findings have important ecological implications, as they suggest that some Gram-positive bacteria may participate in iron cycling in non-sulfidogenic environments despite the absence of a traditional iron respiration pathway. Soluble electron carriers could be released through fermentative processes and slowly consume the pool of bioavailable Fe(III). In effect, environments considered inhospitable to Gram-negative IRB could still be experiencing significant Fe cycling by this type of mechanism, which is mediated by Gram-positive bacteria better adapted to harsh environments.

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SUPPORTING INFORMATION

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

Fig. S1. (a) Cell-free medium (empty circles), lysed (empty triangles), or killed cells (empty squares) are unable to reduce HFO (at certain time points the curves are difficult to distinguish because they are overlapping).

Fig. S2. Fe(II) concentration as a result of Fe(III) citrate (circles) and HFO (diamonds) reduction.

Fig. S3. HFO reduction with pyruvate as electron donor with (filled diamonds) or without (empty circles) H₂ in the headspace.

Fig. S4. Light microscope images showing round bodies (left panel), formed during stationary phase after fermentative growth, and vegetative, exponential phase rod-shaped fermenting *D. reducens* cells (right panel).

Fig. S5. Fe(II) build up as a result of reduction of Fe(III) citrate by *D. reducens* cells from inoculation (diamonds) and by *D. reducens* round bodies (squares).

Fig. S6. Schematic of pyruvate consumption during fermentation.

Fig. S7. Electron micrographs and elemental maps of glass-HFO particle outer surfaces: (a–c) SEM images showing occasional Fe agglomerates (black arrows) on the surface of the glass which were not washed away during the water rinsing; elemental maps showing the distribution of carbon (e), oxygen (f), sodium (g), silica (h), and Fe (i) on the outer surface of the particle in (d), in particular in correspondence of the visible agglomerate (black arrow in (d)).

Fig. S8. Electrons transferred during the chemical reduction of AQDS to AH₂DS or of Fe(III)-citrate to Fe(II) by cell-free spent fermentation medium at T0 (white bars), and after 3 days (gray bars) and 33 days (black bar, only for Fe(II)) of incubation.

Fig. S9. (a) HFO reduction in WLP (no yeast extract (YE), trace elements or vitamins; triangles), and in complete WLP (squares). (b) Pyruvate concentration in the same conditions as (a) and in the absence of HFO, at T0 (black bars) and after 40 h (white bars).

Fig. S10. Fe(III)-citrate reduction by *D. reducens* in fresh WLP medium (circles), spent fermentation medium (diamonds), and spent fermentation medium depleted of S²⁻ (squares).

Table S1. LC-MS/MS analysis of sections of SDS-PAGE gel of total protein extract from *D. reducens* cultures under different conditions: fermentation, fermentation spiked with Fe(III) at mid-exponential phase, and Fe(III) reduction with lactate as electron donor.