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Proof of principle for an engineered microbial biosensor based on *Shewanella oneidensis* outer membrane protein complexes

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ABSTRACT

Shewanella oneidensis is known for its ability to respire on extracellular electron acceptors. The spectrum of these acceptors also includes anode surfaces. Based on this activity, a versatile *S. oneidensis* based biosensor strain was constructed in which electricity production can be modulated. Construction started with the identification of a usable rate-limiting step of electron transfer to an anode. Thereafter, the sensor strain was genetically engineered to produce a protein complex consisting of the three proteins MtrA, MtrB and MtrF. This complex is associated to the outer membrane and most probably enables membrane spanning electron transfer. MtrF is an outer membrane cytochrome that catalyzes electron transfer reactions on the cell surface. Under anoxic conditions, wild type cells do not express MtrF but rather MtrC as electron transferring outer membrane cytochrome. Still, our analysis revealed that MtrF compared to MtrC overexpression is less toxic to the cell which gives MtrF a superior position for biosensor based applications. Transcription of *mtrA*, *mtrB* and *mtrF* was linked up to an inducible promoter system, which positively reacts to rising L-arabinose concentrations. Anode reduction mediated by this strain was linearly dependent on the arabinose content of the medium. This linear dependency was detectable over a wide range of arabinose concentrations. The L-arabinose biosensor presented in this study proves the principle of an outer membrane complex based sensing method which could be easily modified to different specificities by a simple change of the regulatory elements.

1. Introduction

Microbial biosensors use physiological capabilities of microbial cells. These capabilities have to be linked to an output signal that can be detected for instance via optical or electrical devices. The great potential of microbial biosensors lies in the development of inexpensive monitoring techniques that can be operated easily and without sophisticated technical equipment. Many highly specific microbial biosensors rely on the use of genetically modified microorganisms. The organisms usually have a specific promoter region that is responsive to a certain analyte. The promoter region triggers the expression of a reporter gene. The corresponding protein delivers directly or indirectly a measurable signal. Measuring techniques for these rather specific systems are often based on the detection of optical stimuli (luminescence, color changes or fluorescence). The other major group of microbial biosensors is based on electrochemical measurements (Pasco et al., 2011; Su et al., 2011). Among these, amperometry is the most common technique used in biosensors. The strategy is to apply a certain potential to working and reference electrode and to detect and quantify a current signal that results from oxidation or reduction of an electroactive compound. This technique is widely used to measure the biological oxygen demand of solutions that contain a mixture of reduced organic carbon sources (Kumlanghan et al., 2008; Nakamura et al., 2007). These measurements rely on aerobic respiration, since microbes couple the oxidation of suitable reduced organic carbon sources to the reduction of oxygen to water.

Recently microbial fuel cell (MFC) based systems were introduced to the field of biosensors (Gil et al., 2003; Kim et al., 2003). MFCs are usually known as an approach to harvest electricity. This electricity is produced via the catabolic, respiratory oxidation of electron donors which is coupled to anode reduction. So-called exoelectrogenic microorganisms transfer electrons directly or via a mediator to an anode. This can be easily measured and quantified since a current is produced as a result of this form of anaerobic respiration. To date, it was shown in several studies that MFCs can be applied to measure the biological oxygen demand (Chang et al., 2004; Di Lorenzo et al., 2009; Gil et al., 2003; Kim et al., 2003; Kumlanghan et al., 2007). The same technique can also be used to quantify specific analytes if the solution that is measured contains only one compound that can be oxidized by the microbes or if the microbes have a rather restricted spectrum of usable electron

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donors. A slight modification of the above-mentioned principles leads to techniques for detection and quantification of toxic compounds. These compounds interfere negatively with the respiratory capabilities of microorganisms. In other words, microbes will need more time to respire available oxygen in the presence of an oxidizable carbon source if toxic compounds like phenol or chromium inhibit them (Davila et al., 2011; Kim et al., 2007). To our knowledge, the only MFC based biosensors that claim to be specific for an analyte depend directly on the carbon metabolism of the microorganism used. Tront et al. (2008a, 2008b) for instance used *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 cells to quantify acetate and lactate concentrations, respectively. Still, recent analysis showed that these organisms are not as limited in their spectrum of oxidizable carbon sources as formerly believed (Call and Logan, 2011; Yang et al., 2006) which questions the specificity of the biosensor if operated in a mixture of organic carbon sources.

In this study, we present a genetically engineered *S. oneidensis* strain as a platform for a microbial biosensor. The ability of this strain to produce current was set under control of a heterologous promoter in a synthetic biology approach. The biosensor is highly specific and can be adapted to many different analytes by the insertion of new promoter regions. Its performance was evaluated under defined anode reducing conditions controlled by a potentiostat. The sensor is based on *S. oneidensis*, one of the best-characterized model organisms for anode respiration besides *G. sulfurreducens*. This non-pathogenic facultative anaerobic γ-proteobacterium is closely related to *Escherichia coli* and shows a good genetic tractability (Hau and Gralnick, 2007; Richter et al., 2012). *S. oneidensis* is known for its ability to respire insoluble metal oxides which also enables electron transfer to an anode without addition of an external mediator. This ability to reduce an anode is certainly not due to an evolutionary adaptation but rather to an unspecificity of the terminal reductases. These enzymes bring respiratory electrons into contact with extracellular electron acceptors and it is not important whether this electron acceptor is a graphite electrode, hydrous ferric oxide or Fe(III) citrate. Hence, the components of the electron transport chain to an anode and to iron oxides are either highly similar or even identical. Therefore, most of the experiments described in this study were conducted first with ferric iron as electron acceptor and at certain stage were expanded to anode-reduction experiments.

The organism's ability to respire on insoluble electron acceptors is based on an extended respiratory chain that transports electrons to the cell surface (Richter et al., 2012). The current model of the extended respiratory chain in *S. oneidensis* is depicted in Fig. S1. It consists mainly of multiheme *c*-type cytochromes. They are required for transport of respiratory electrons through periplasm and outer membrane and the final reduction of extracellular electron acceptors. This final reduction step seems to be rate limiting in most cases (Ross et al., 2009). It is catalyzed in *S. oneidensis* by outer membrane cytochromes (OMC). Evidence was provided that two decaheme OMCs are of outstanding importance for anode respiration (Bretschger et al., 2007; Meitl et al., 2009). These OMCs are called OmcA (**o**uter **m**embrane **c**ytochrome **A**) and MtrC (**m**e**t**al **r**educing protein **C**). The latter one was shown to be part of an electron transferring protein complex that spans the outer membrane (Hartshorne et al., 2009; Richter et al., 2012). Therefore, anode respiration necessitates the presence of MtrC. An additional gene for an outer membrane cytochrome, *mtrF*, was shown to encode for a functional protein that can rescue the phenotype of *mtrC* deletion mutants (Bücking et al., 2010; Clarke et al., 2011). Still, *mtrF* seems to be expressed only under microoxic conditions (McLean et al., 2008). As mentioned above, OMCs catalyze direct reduction of metal oxides and anodes but they are furthermore necessary for the reduction of endogenous or exogenous electron shuttles that facilitate extracellular electron transfer (Coursolle et al., 2010; Jiang et al., 2010; Marsili et al., 2008; Ross et al., 2009).

The specific engineered biosensor presented here is based on the construction of an operon composed of the genes *mtrF*, *mtrA* and *mtrB*. This operon was inserted in the genome of a recently published strain that is devoid of any gene encoding an outer membrane cytochrome (Bücking et al., 2010). The new operon was linked up to a P_{BAD} promoter. We show that modulation of the operon expression levels is a powerful tool to adjust ferric iron reduction capacity and consequently anode reduction performance.

2. Materials and methods

Chemicals and biochemicals were obtained from Sigma-Aldrich (Munich, Germany), Roth (Karlsruhe, Germany) and Promega (Mannheim, Germany). Enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany).

2.1. Growth conditions and media

All microorganisms used in this study are listed in Table S1. *E. coli* strains were grown in LB medium at 37 °C, *S. oneidensis* strains were grown aerobically at 30 °C in LB medium or under anoxic conditions in minimal medium, supplemented with lactate (50 mM) as electron donor and carbon source and 50 mM Fe(III) citrate or 100 mM fumarate as electron acceptor as described previously (Schuetz et al., 2009). If necessary, kanamycin (50 μg ml^{-1}) and arabinose (0.01–2 mM) was added to the medium. For electrochemical experiments, the growth medium was a modified PBS buffer (2.7 mM KCl, 1.76 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, 9 mM $(NH_4)_2SO_4$, 1 mM Mg_2SO_4, 0.1 mM $CaCl_2$) containing 50 mM lactate as electron donor and carbon source, 1 g l^{-1} casamino acids, and trace elements (5 μM $CoCl_2$, 0.2 μM $CuSO_4$, 57 μM H_3BO_3, 5.4 μM $FeCl_2$, 1.3 μM $MnSO_4$, 67.2 μM Na_2EDTA, 3.9 μM Na_2MoO_4, 1.5 μM Na_2SeO_4, 5 μM $NiCl_2$, and 1 μM $ZnSO_4$).

2.2. Construction of S. oneidensis MTR-FAB mutant

All used primers and plasmids are listed in Tables S2 and S3 respectively. The gene *mtrF* containing a C-terminal *Strep*-tag® was introduced into the existing strain ΔOMC in front of the genes *mtrA* and *mtrB* (Bücking et al., 2010). Primers 1 and 2 were used to amplify a 500bp region upstream of the insertion site in ΔOMC and *mtrF* using plasmid DNA from $P_{BAD}mtrF_{strep}$ as a template (Bücking et al., 2010). Primers 3 and 4 were used to amplify a 500bp region downstream of the insertion site. The resulting fragments were fused by a further PCR step utilizing Primers 1 and 4 which also created two additional 50bp overlaps at the ends of the fused fragment. These overlaps were homologs to the ends of a pMQ150 plasmid which was linearized with BamHI and NcoI (New England Biolabs, Frankfurt am Main, Germany). All created PCR fragments and digested plasmids were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany) according to the manufacturer's instructions. Equimolar amounts of the merged fragment and the linearized pMQ150 plasmid (overall concentration 100 ng DNA) were recombined using an isothermal assembly method (Gibson et al., 2009). 50 ng of the resulting plasmid pMQ150-*mtrFAB* were transformed into *E. coli* S17, which was then used as conjugal donor strain for mating with *S. oneidensis* ΔOMC. Markerless mutants harboring an *mtrF* insertion in the genome were gained as described previously (Bücking et al., 2010). The introduced DNA of the resulting mutant,

designated *S. oneidensis* MTR-FAB, was sequenced by GATC Biotech (Constance, Germany).

2.3. Membrane-preparation, SDS-PAGE, heme-staining, and western-blotting

For membrane-preparation cells were harvested via centrifugation at 3000 *g* and 4 °C for 10 min, resuspended in 0.1 M HEPES (pH 7.4, 10% glycerol) containing 0.1 mg ml^{-1} DNase I, and passed through a French pressure cell (Thermo Fisher Scientific, Langensebold, Germany; American Instruments Company, Maryland, USA) at 137 MPa. The cell lysate was spun at 3000 *g* and 4 °C for 5 min to remove non-disrupted cells. The supernatant was centrifuged at 208,000*g* and 4 °C for 90 min to separate the soluble from the membrane fraction. Protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard. For quantification of protein concentrations in cell suspensions, 0.2 mM NaOH was added to the suspensions prior to a 10 min incubation at 95 °C.

Proteins were separated on polyacrylamide gels according to Laemmli (1970). Heme proteins were visualized by peroxidase staining (Thomas et al. 1976). Proteins containing a C-terminal *Strep*-tag® were detected on a western blot using a primary strep-tag antibody (Qiagen, Hilden, Germany) and a secondary alkaline phosphatase labeled antibody (Sigma-Aldrich, Munich). The blot was developed using the AP conjugate detection kit (Biorad, Munich).

2.4. Fe(III) citrate reduction experiments

Cells were pre-grown overnight in minimal medium under anoxic conditions with fumarate as electron acceptor and induced with 0–2 mM arabinose. Cells were then washed twice, resuspended to a final OD_{600} of 0.4 and 500 μl of this cell suspension were used to inoculate 100 ml anoxic Fe(III) citrate medium. Reduction of Fe(III) citrate was determined by measuring ferrous iron concentrations with the ferrozine reagent (Stookey, 1970). All experiments were conducted in independent triplicates. The maximal reduction rate was determined by a non-linear curve fit done with GraphPad Prism software.

2.5. Microbial biosensor experiments

The electrochemical setup used in this study was previously described by Kloke et al. (2010). It features an anode and cathode chamber harboring the working and the counter electrode respectively. Both chambers had a working volume of 25 ml and were separated by a Fumion F950 membrane (Quintech, Göppingen, Germany). A saturated calomel reference electrode (SCE) (Sensortechnik Meinsberg, Ziegra-Knobelsdorf, Germany) was separated from the anode compartment by another Fumion membrane. Graphite felt (SGL Carbon SE, Wiesbaden, Germany) was used as a material for working and counter electrodes. Potentials measured at the working electrode against the SCE were converted to normal hydrogen electrode (NHE) potentials by addition of 237.9 mV.

Cells were pre-grown over night under anoxic conditions with fumarate as electron acceptor. Cultures were thereafter spun down and washed twice with anoxic medium that did not contain an electron acceptor to remove residual fumarate. In all experiments the anode chamber contained 25 ml of PBS–lactate-medium with arabinose concentrations as indicated, whereas the cathode chamber contained 25 ml of PBS medium lacking lactate. Experiments were initiated by adding cells to the anode compartment using a starting $OD_{600\ nm}$ of 0.4. The whole setup was connected to a potentiostat (Pine Instruments), which controlled the long measurement protocol that contained two phases: first, a constant current was applied (conditioning period, 278 nA cm^{-2}) until a stable potential was reached. Then, this current was continuously increased (current sweep phase) with a rate of 2.88 μA h^{-1} until 120 μA were reached. The limiting current density that is characterized by a steep rise in potential was defined to be +750 mV vs. NHE according to earlier work (Bücking et al., 2010).

A short measurement protocol was developed for biosensor applications. Here, cells were also pre-grown over night under anoxic conditions but were not induced with arabinose. Operation of the biosensor was similar but consisted only of the first phase with a constant current of 444 nA cm^{-2}. The anode compartment was continuously flushed with nitrogen gas to maintain anoxic conditions. Additional terminal electron acceptors were not added. All biosensor experiments were conducted in independent duplicates or triplicates at a constant temperature of 30 °C.

3. Results

3.1. Finding the right outer membrane cytochrome

In a previous study it was shown that expression of either MtrC or MtrF in an outer membrane cytochrome deficient *S. oneidensis* mutant (strain ΔOMC) can restore the ability to respire on iron oxides and anode surfaces (Bücking et al., 2010). In contrast, expression of OmcA, which is present in the outer membrane of wild type cells at high concentrations, did not rescue the ΔOMC mutant phenotype. Therefore, it was tested first whether and to what extent MtrC and MtrF can be overexpressed in the ΔOMC strain. This information was necessary for the construction of the biosensor since the window in which electron transfer can be modulated by OMC expression defines the spectrum of concentrations that can be assessed by the biosensor itself. Arabinose inducible plasmids carrying either a copy of the *mtrC* (strain JG138) or the *mtrF* (JG139) gene were transformed into the ΔOMC strain. Thereafter Fe(III) citrate reduction was recorded in batch cultures at varying levels of

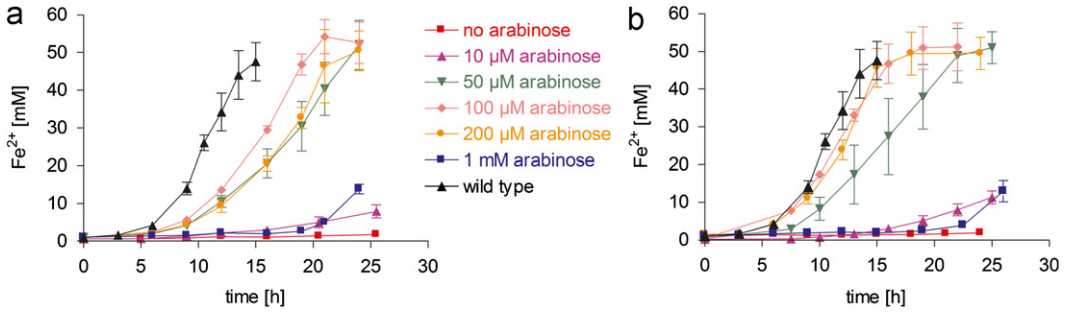


Fig. 1. Iron reduction with ΔOMC cells complemented with pBad*mtrC*$_{strep}$ (a) and pBad*mtrF*$_{strep}$ (b) plasmids. Fe(II) production was monitored over time using the ferrozine assay. Mutant strains were induced with arabinose as indicated. Symbols refer to means of triplicate experiments, error bars indicate standard deviation.

arabinose in the medium ranging from 0–1 mM (Fig. 1). Fastest reduction was observed with 0.1 mM and 0.2 mM arabinose for strain JG138 and JG139, respectively. Surprisingly, a higher induction lowered the ferric iron reduction rate and full induction nearly prohibited reduction at all (Fig. 1). Toxic effects of the expression might account for this phenomenon. The concentration windows of the inducer that were proportional to the reduction rate were rather narrow for both strains (for JG138 from 0 to 100 μM and for JG139 from 0 to 200 μM). Still, it was twofold larger if MtrF instead of MtrC was used. A disadvantage of the strains described so far is that the components of the outer membrane spanning complex are not produced in equal amounts. *mtrA* and *mtrB* are encoded in one copy per cell in the genome, while *mtrC* and *mtrF* were expressed from a low copy plasmid and probably have a 10–30-fold higher copy number. Additionally the function of these strains in a biosensor would rely on plasmids which need to be maintained in the cell through a continuous addition of an antibiotic.

3.2. Genomic insertion widens the usable induction window

A new *S. oneidensis* strain was constructed to overcome the limitations mentioned above and to integrate the information gained from the expression experiments. Therefore, a ΔOMC strain backbone was used and the genes encoding *mtrF*, *mtrA* and *mtrB* were placed in a row behind a P_{BAD}-promoter in the genome resulting in strain MTR-FAB (JG410). As Fig. 3 indicates, the concentrations of MtrF and MtrA increase proportionally with the induction level. Although the expression of MtrB could not be shown directly due to the absence of a specific antibody, the protein is very likely to be present in similar amounts since it is part of the same operon (Fig. 2).

The Fe(III) citrate reduction rate of MTR-FAB responded to a much greater range of inducer concentrations as compared to the above described experiments with plasmid encoded copies of *mtrF*. Fastest ferric iron reduction rates were reached using 1 mM arabinose (Fig. 4). Further increase in arabinose concentration did not increase the maximal reduction rate and the onset of reduction was delayed, probably due to a negative effect from protein overproduction. Interestingly, the maximal reduction rate of MTR-FAB with an induction of 1 mM arabinose was 1.8-fold higher than that of the wild type.

3.3. Biosensor performance of MTR-FAB

The ultimate goal of this study was to construct a biosensor strain that can be used in a MFC based system. Hence, it was asked whether the dependency of ferric iron reduction on inducer level would directly convert to a dependency of anodic current production on inducer concentration in a bioelectrical system. The setup was controlled by a potentiostat to compare wild type and MTR-FAB strain under different induction levels. While at first a fixed low current was applied to allow for anode colonization, the current was then increased linearly and the potential was measured. The current density at which bacteria failed to provide sufficient quantities of electrons to sustain a given current abstraction is characterized by a steep increase in slope of the current density–potential plot (to an average of 43.4 ± 5.9 μA cm^2/mV) after an inflection point at 750 mV (Fig. 5a). The current at 750 mV is defined as the limiting current density (LCD) which is a characteristic for each strain (Bücking et al., 2010).

The LCDs (means of triplicate measurements) of strain MTR-FAB increased with the induction level significantly starting from 0.1 mM and reaching a maximum at 2 mM arabinose. At this point, the LCD of MTR-FAB is 1.13-fold higher than the one of the wild type (Fig. 5b). The relation between induction level and LCD is linear over a wide range of inducer concentrations. The curve levels

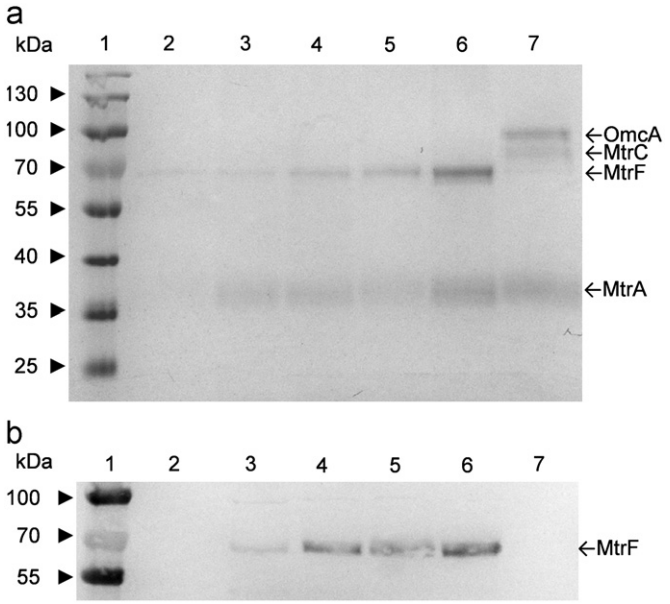


Fig. 3. SDS-PAGE of membrane fractions from strain *S. oneidensis* MTR-FAB and wild type. (a) Heme acitivity staining (b) Western Blot with anti-strep antibody to detect $MtrF_{strep}$. Lane 1: protein marker; lanes 2–6: *S. oneidensis* MTR-FAB with 100 μM, 200 μM, 500 μM, 1 mM, and 2 mM arabinose, correspondingly; lane 7: *S. oneidensis* wild type. The faint band in Fig. 4a, lane 2, which does not correlate to a positive signal in the corresponding western is most likely a minor contamination with the periplasmic *c*-type cytochrome FccA.

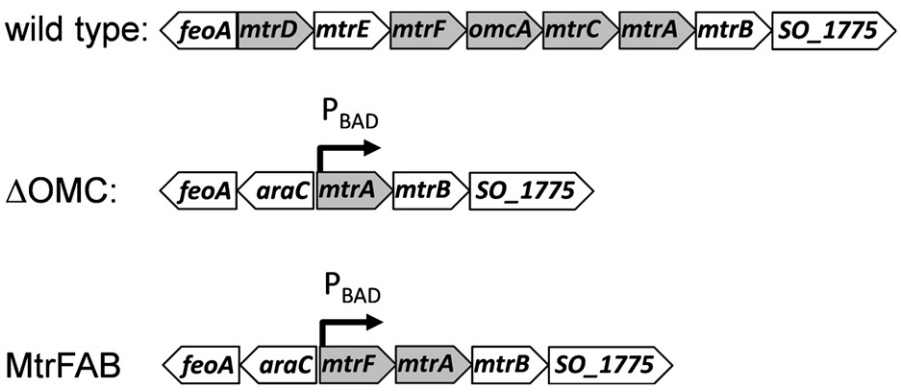


Fig. 2. Schematic view of the *mtr*-gene clusters of *S. oneidensis* MR-1 and mutant strains. Genes coding for *c*-type cytochrome proteins are depicted in gray. The gene *araC* codes for the repressor/activator protein AraC that is interacting with the P_{BAD} promoter which is symbolized by a black arrow.

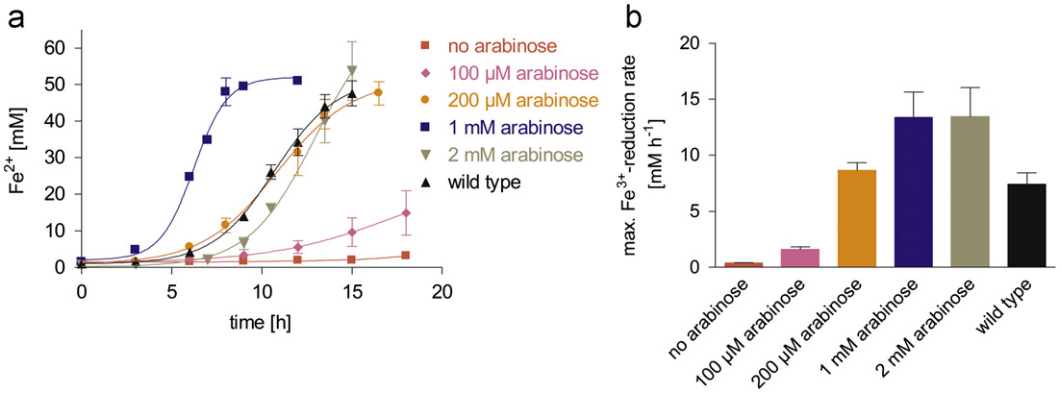


Fig. 4. Fe(III)-citrate reduction and maximal reduction rates by strain MTR-FAB (JG410). (a) Fe(II) production was monitored over time using the ferrozine assay. MTR-FAB strain was induced with arabinose as indicated. Symbols refer to means of triplicate experiments, error bars indicate standard deviation. (b) Maximal reduction rates during the course of the experiment calculated for each biological replicate with GraphPad Prism 4. Error bars represent standard deviations

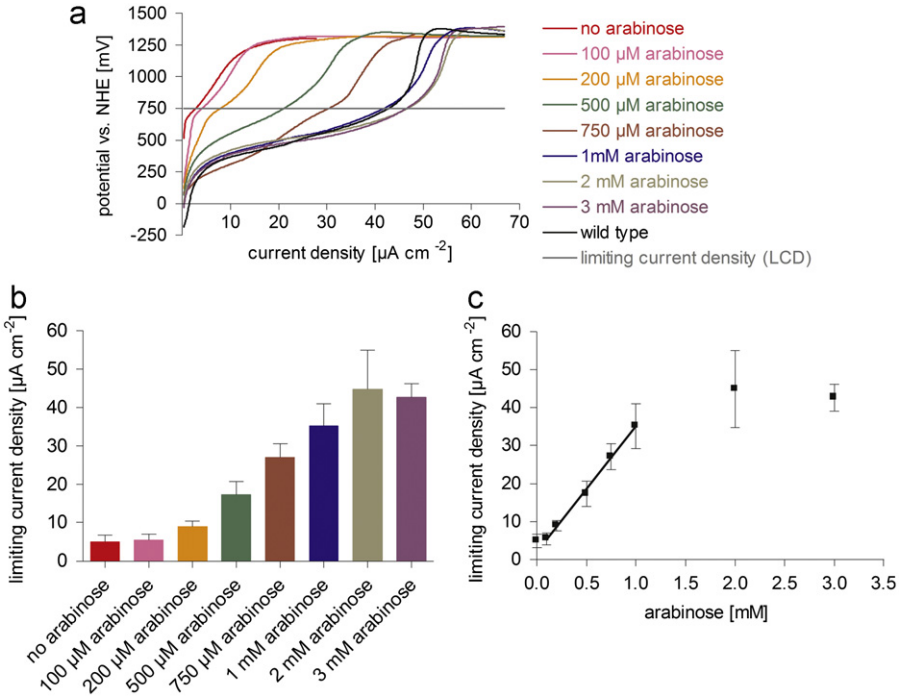


Fig. 5. Biosensor experiments with MTR-FAB. (a) The current density at the anode was increased continuously and the reached potentials were recorded. Solid lines are representative plots. (b) Limiting current density was determined by measuring the current density when the potential reached +750 mV vs. NHE. Error bars indicate standard deviation from triplicate experiments. (c) Arabinose concentration plotted against limiting current density. A linear dependence from 0.1–1 mM is illustrated by a regression curve ($y=32.99x+1.98$; $r^2=0.92$).

off when more than 1 mM arabinose was added to the medium. Still, the linear dependence between 0.1 and 1 mM arabinose which is illustrated by a linear regression curve (Fig. 5c, $y=32.99x+1.98$, $r^2=0.92$; y=LCD; x=arabinose concentration) allows for the use of this system for sensor applications (Fig. 5c). A measured current value could then be traced back to an unknown inducer concentration.

3.4. Decreasing the measurement period

A faster biosensor measurement protocol was developed to show that the different induction levels could be sensed without performing a time consuming current–sweep experiment. Therefore the potential generated by the bacteria was compared at a fixed current density. This experiment was performed using inducer concentrations of 0, 0.5, and 1 mM. To be closer to a putative application, the experiment was conducted with cells that were not precultured with arabinose as inducer. Consequently, these cells were expected to show a delay in Mtr-FAB expression which is consistent with the almost identical progression of the determined potentials of all three setups during the first 0.75 h (Fig. 6a). Beyond that period the potential of the induced setups started to drop whereas that of the non-induced setup was barely affected. After 2 h of chronopotentiometry a difference between all three induction levels was clearly visible. Compared to the non-induced setup those with induction levels of 0.5 and 1 mM arabinose reached a 1.13- and 1.75-fold lower potential, respectively (Fig 6b). Thereby, the described drops of the potentials seemed to be related to the increasing number of expressed Mtr-FAB-complexes in this phase. Due to these results this method could be a promising approach for a relatively quick and specific detection of so far not measurable compounds. (Scheme 1).

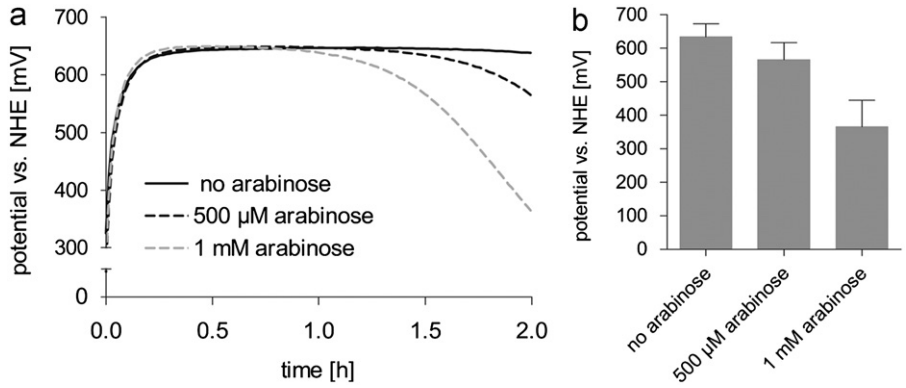
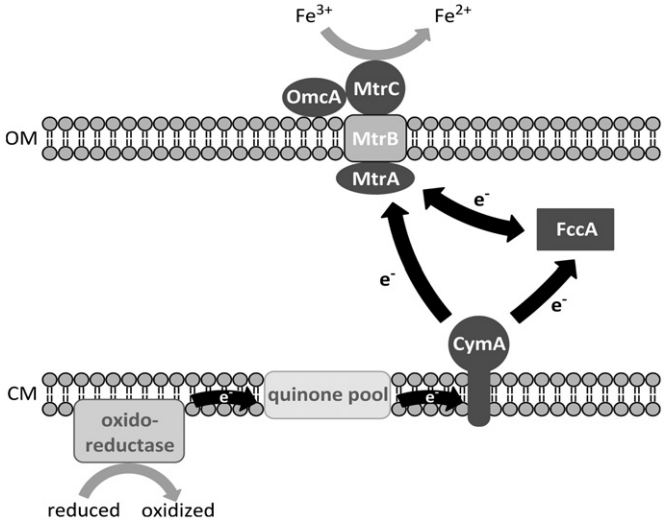


Fig. 6. Development of the potential during a short conditioning period. (a) The development of the anode potentials for three different induction levels is shown. The lines represent mean values of the experiments. (b) The columns represent the potential that was reached 2 h after starting the experiment. They were calculated from duplicate experiments. Error bars represent deviations from the mean.

4. Discussion

In this article, we showed the development of a platform for a versatile microbial biosensor using a genetically modified *S. oneidensis* strain. This sensor system uses MFC technology which has been successfully used before for the determination of biological oxygen demands for instance and show high longevity (Chang et al., 2004; Kim et al., 2003). Our approach is based on the controlled expression of proteins that are necessary for electron transfer through the outer membrane and onto solid phase extracellular electron acceptors. To our knowledge, this is the first report of a bioanode based biosensor, which responds to a single substance with high specificity. *S. oneidensis* was before presented as a potential biosensor organism in a study by Tront et al. (2008b). In this study the authors measure current production during the consumption of lactate, which is the electron and carbon source for the organism. This lactate biosensor comes along with a number of disadvantages compared to the here described technology. First, the spectrum of substances that can be detected is limited by the carbon and electron sources that can be used by the organism (lactate, N-acetylglucosamine, formate, and hydrogen). The here described system is limited only by the amount of regulatory machineries and the potential of substances to diffuse or be transported into the cytoplasm (arabinose for instance is not a carbon or electron source for *S. oneidensis*). Therefore, the spectrum of analytes that can be sensed will be way above the four carbon and electron sources of the organism. Second, the response time of the sensor as described by Tront et al. was up to 19 h, which is way above the 2 h that can be reached with the fast measurement protocol described in the last part of the results section. Last but not least, the detection limit for a certain lactate concentration was 3 mM. This is way above the 0.1 mM for arabinose that can be reached with the here described system. In summary, via the here presented concept it is possible to sense a specific compound which is not the substrate over a well defined range of concentrations (0.1–1 mM arabinose) with high accuracy ($R^2=0.92$). A further strength of our concept lies in its simplicity which can result in very cheap sensor constructs compared to other systems which require elaborated technical equipment (Ha et al., 2012).

It was interesting to observe that the developed strain reduced Fe(III) citrate with reduction rates that were dependent on the inducer concentration. At a certain concentration the rates did not increase further but rather reached a plateau. This plateau was probably reached because the capabilities of the promoter were maxed out by inducer concentrations of more than 1 mM arabinose in the medium. In other words, the expression level could not have been increased beyond that point. Another possibility is that



Scheme 1. Current model of the extended respiratory chain of S. oneidensis. c-type cytochrome proteins are depicted in dark grey. OM= outer membrane, CM= cytoplasmic membrane.

maturation of the *c*-type cytochromes MtrF and MtrA or membrane insertion of MtrB was limiting. These latter points might especially contribute to the toxic effects that were observed when MtrC or MtrF were expressed from plasmid encoded copies of the corresponding genes. Still, the developed MTR-FAB strain reduced Fe(III) citrate faster than wild type cells. This means that the ecological niche of *S. oneidensis* might not select for the fastest ferric iron reducer but that other factors contribute most probably as well. These facts suggest that there are further possibilities to accelerate iron reduction in engineered strains, which will provide an interesting field for future research.

It was surprising to see, that most but not all results from ferric iron reduction experiments were transferrable to bioelectrical systems. Certainly, it was the most important point to show that current production could be modulated by the inducer concentration in the medium. This is the prerequisite for a quantitative biosensor. The experiments presented here clearly demonstrate that MTR-FAB can be operated as a microbial biosensor and future experiments will expand the spectrum of detectable substances. This spectrum depends only on available promoter systems and the capability of the target compounds to enter a *Shewanella* cell. Still, the effect of Mtr-FAB expression on maximum current production was not as pronounced as the observed impact on the ferric iron reduction rate. The MTR-FAB strain produced more

current than the wild type but only 1.13-fold more while ferric iron reduction was 1.8-fold accelerated. So far, it is not possible to undoubtedly specify the reasons for this difference. It is known that Fe(III) citrate is a substance that is reduced extracellularly and that its reduction necessitates MtrF in the MTR-FAB strain. This is also true for anode reduction by MTR-FAB. We hypothesize that the availability of the two electron acceptors causes the observed difference. Fe(III) citrate is a soluble or colloidal electron acceptor that is unable to pass the outer membrane (Dobbin et al., 1996; Gescher et al., 2008; Pitts et al., 2003). Still, one could imagine that Fe(III) citrate is by far more available to outer membrane cytochromes at the cell surface reaching the bacterial cell from all sides than a graphite felt anode which is a solid substance. Hence, only a subfraction of the produced Mtr-FAB complexes is able to interact with the anode surface. This fraction is most likely smaller than the fraction that can interact with Fe(III) citrate as a diffusible electron acceptor. Therefore, we hypothesize that a part of the protein complexes cannot interact with the anode surface and therefore does not contribute to current production. Consequently, the effect of Mtr-FAB expression cannot be as pronounced in anode reduction as compared to Fe(III) citrate reduction.

5. Conclusions

This study describes the development of an engineered microbial biosensor. An arabinose inducible promoter system was used here to conduct proof of principle experiments. The results display clearly that the current production depends on the addition of arabinose in a linear fashion. The developed strain can be easily adapted to detect and quantify a number of other compounds, which would only require a simple exchange of the promoter system. The measurement period was reduced to less than 2 h. This will be sufficient for a multitude of applications. To our knowledge this is the first report about a bioanode based biosensor that is responsive to a specific analyte which is not used as carbon or electron source. It can be adapted to various specifications by exchanging the promoter region using established protocols.

Disclosure statement

The authors disclose that an accompanying European patent was filed. The patent application (EP 12183209.1) comprises the described biosensor technology and additionally the genetically engineered strain JG410, which was deposited in the German Collection of Microorganisms and Cell Cultures (DSM 26201).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.03.010.

References

Bradford, M.M., 1976. Analytical Biochemistry 72, 248–254.

Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., Zhou, J., Beliaev, A.S., Bouhenni, R., Saffarini, K.H., Mansfeld, F., Kim, B.H., Fredrickson, J.K., Nealson, K.H., 2007. Applied and Environmental Microbiology 73 (21), 7003–7012.

Bücking, C., Popp, F., Kerzenmacher, S., Gescher, J., 2010. FEMS Microbiology Letters 306 (2), 144–151.

Call, D.F., Logan, B.E., 2011. Applied and Environmental Microbiology 77 (24), 8791–8794.

Chang, I.S., Jang, J.K., Gil, G.C., Kim, M., Kim, H.J., Cho, B.W., Kim, B.H., 2004. Biosensors and Bioelectronics 19 (6), 607–613.

Clarke, T.A., Edwards, M.J., Gates, A.J., Hall, A., White, G.F., Bradley, J., Reardon, C.L., Shi, L., Beliaev, A.S., Marshall, M.J., Wang, Z., Watmough, N.J., Fredrickson, J.K., Zachara, J.M., Butt, J.N., Richardson, D.J., 2011. Proceedings of the National Academy of Sciences United States of America 108 (23), 9384–9389.

Coursolle, D., Baron, D.B., Bond, D.R., Gralnick, J.A., 2010. Journal of Bacteriology 192 (2), 467–474.

Davila, D., Esquivel, J.P., Sabate, N., Mas, J., 2011. Biosensors and Bioelectronics 26 (5), 2426–2430.

Di Lorenzo, M., Curtis, T.P., Head, I.M., Scott, K., 2009. Water Research 43 (13), 3145–3154.

Dobbin, P.S., Warren, L.H., Cook, N.J., McEwan, A.G., Powell, A.K., Richardson, D.J., 1996. Microbiology 142, 765–774.

Gescher, J.S., Cordova, C.D., Spormann, A.M., 2008. Molecular Microbiology 68 (3), 706–719.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Nature Methods 6 (5), 343–345.

Gil, G.C., Chang, I.S., Kim, B.H., Kim, M., Jang, J.K., Park, H.S., Kim, H.J., 2003. Biosensors and Bioelectronics 18 (4), 327–334.

Ha, J.S., Gam, J., Choi, S.L., Oh, K.H., Ro, H.S., Song, J.J., Shin, C.S., Lee, S.G., 2012. Biotechnology Progress 28 (5), 1376–1383.

Hartshorne, R.S., Reardon, C.L., Ross, D., Nuester, J., Clarke, T.A., Gates, A.J., Mills, P.C., Fredrickson, J.K., Zachara, J.M., Shi, L., Beliaev, A.S., Marshall, M.J., Tien, M., Brantley, S., Butt, J.N., Richardson, D.J., 2009. Proceedings of the National Academy of Sciences United States of America 106 (52), 22169–22174.

Hau, H.H., Gralnick, J.A., 2007. Annual Review of Microbiology 61, 237–258.

Jiang, X., Hu, J., Fitzgerald, L.A., Biffinger, J.C., Xie, P., Ringeisen, B.R., Lieber, C.M., 2010. Proceedings of the National Academy of Sciences United States of America 107 (39), 16806–16810.

Kim, B.H., Chang, I.S., Gil, G.C., Park, H.S., Kim, H.J., 2003. Biotechnology Letters 25 (7), 541–545.

Kim, M., Hyun, M.S., Gadd, G.M., Kim, H.J., 2007. Journal of Environmental Monitoring 9 (12), 1323–1328.

Kloke, A., Rubenwolf, S., Bücking, C., Gescher, J., Kerzenmacher, S., Zengerle, R., von Stetten, F., 2010. Biosensors and Bioelectronics 25 (12), 2559–2565.

Kumlanghan, A., Kanatharana, P., Asawatreratanakul, P., Mattiasson, B., Thavarungkul, P., 2008. Enzyme and Microbial Technology 42 (6), 483–491.

Kumlanghan, A., Liu, J., Thavarungkul, P., Kanatharana, P., Mattiasson, B., 2007. Biosensors and Bioelectronics 22 (12), 2939–2944.

Laemmli, U.K., 1970. Nature 227 (5259), 680–685.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., Bond, D.R., 2008. Proceedings of the National Academy of Sciences United States of America 105 (10), 3968–3973.

McLean, J.S., Pinchuk, G.E., Geydebrekht, O.V., Bilskis, C.L., Zakrajsek, B.A., Hill, E.A., Saffarini, D.A., Romine, M.F., Gorby, Y.A., Fredrickson, J.K., Beliaev, A.S., 2008. Environmental Microbiology 10 (7), 1861–1876.

Meitl, L.A., Eggleston, C.M., Colberg, P.J.S., Khare, N., Reardon, C.L., Shi, L., 2009. Geochimica et Cosmochimica Acta 73 (18), 5292–5307.

Nakamura, H., Suzuki, K., Ishikuro, H., Kinoshita, S., Koizumi, R., Okuma, S., Gotoh, M., Karube, I., 2007. Talanta 72 (1), 210–216.

Pasco, N.F., Weld, R.J., Hay, J.M., Gooneratne, R., 2011. Analytical and Bioanalytical Chemistry 400 (4), 931–945.

Pitts, K.E., Dobbin, P.S., Reyes-Ramirez, F., Thomson, A.J., Richardson, D.J., Seward, H.E., 2003. Journal of Biological Chemistry 278 (30), 27758–27765.

Richter, K., Schicklberger, M., Gescher, J., 2012. Applied and Environmental Microbiology 78 (4), 913–921.

Ross, D.E., Brantley, S.L., Tien, M., 2009. Applied and Environmental Microbiology 75, 5218–5226.

Schuetz, B., Schicklberger, M., Kuermann, J., Spormann, A.M., Gescher, J., 2009. Applied and Environmental Microbiology 75 (24), 7789–7796.

Stookey, L.L., 1970. Analytical Chemistry 42, 779781.

Su, L.A., Jia, W.Z., Hou, C.J., Lei, Y., 2011. Biosensors and Bioelectronics 26 (5), 1788–1799.

Thomas, P.E., Ryan, D., Levin, W., 1976. Analytical Biochemistry 75 (1), 168–176.

Tront, J.M., Fortner, J.D., Ploetze, M., Hughes, J.B., Puzrin, A.M., 2008a. Biosensors and Bioelectronics 24 (4), 586–590.

Tront, J.M., Fortner, J.D., Ploetze, M., Hughes, J.B., Puzrin, A.M., 2008b. Biotechnology Letters 30 (8), 1385–1390.

Yang, C., Rodionov, D.A., Li, X., Laikova, O.N., Gelfand, M.S., Zagnitko, O.P., Romine, M.F., Obraztsova, A.Y., Nealson, K.H., Osterman, A.L., 2006. Journal of Biological Chemistry 281 (40), 29872–29885.