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Shewanella oneidensis in a lactate-fed pure-culture and a glucose-fed co-culture with *Lactococcus lactis* with an electrode as electron acceptor

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ABSTRACT

Bioelectrochemical systems (BESs) employing mixed microbial communities as biocatalysts are gaining importance as potential renewable energy, bioremediation, or biosensing devices. While we are beginning to understand how individual microbial species interact with an electrode as electron donor, little is known about the interactions between different microbial species in a community: sugar fermenting bacteria can interact with current producing microbes in a fashion that is either neutral, positively enhancing, or even negatively affecting. Here, we compare the bioelectrochemical performance of *Shewanella oneidensis* in a pure-culture and in a co-culture with the homolactic acid fermenter *Lactococcus lactis* at conditions that are pertinent to conventional BES operation. While *S. oneidensis* alone can only use lactate as electron donor for current production, the co-culture is able to convert glucose into current with a comparable coulombic efficiency of ~17%. With (electro)-chemical analysis and transcription profiling, we found that the BES performance and *S. oneidensis* physiology were not significantly different whether grown as a pure- or co-culture. Thus, the microbes worked together in a purely substrate based (neutral) relationship. These co-culture experiments represent an important step in understanding microbial interactions in BES communities with the goal to design complex microbial communities, which specifically convert target substrates into electricity.

1. Introduction

The ability to respire with solid terminal electron acceptors, such as mineral oxides or electrodes, has positioned *Shewanella oneidensis* MR-1 as a model organism for microbial fuel cell (MFC) or – more generally – bioelectrochemical system (BES) research (Bretschger et al., 2007; Gorby et al., 2006; Kim et al., 1999, 2002; Marsili et al., 2008; Nealson et al., 2002). In the anode compartment of BESs, microorganisms transfer electrons, which result from the breakdown of organic substrates, to the anode (i.e., anaerobic respiration with a solid electron acceptor). Such transfer of electrons is highly promising for novel applications in wastewater treatment, bioremediation, and biosensing. All known mechanisms for microbial extracellular respiration are proposed to be employed by *S. oneidensis*: (i) direct electron transfer with outer membrane redox-proteins (Kim et al., 1999; Myers and Myers, 2001), possibly with the help of conductive appendages (Gorby et al., 2006); and (ii) mediated electron transfer with microbially produced soluble redox-compounds (Marsili et al., 2008; von Canstein et al., 2008). Besides *S. oneidensis*, the direct electron-transfer bacterium *Geobacter sulfurreducens* and the mediator (phenazine) producer *Pseudomonas aeruginosa* represent important model organisms for the functional investigation of extracellular electron transfer in bioelectrochemical systems (often referred to as anode respiring bacteria [ARB]).

Shewanella's typical electron donor for anaerobic respiration with solid electron acceptors is lactate, which is oxidized to acetate, carbon dioxide, and four electrons. Recent studies have shown that oxygen promotes the utilization of a wider spectrum of carbon sources by *S. oneidensis* for electric current generation (e.g., acetate) (Biffinger et al., 2008; Ringeisen et al., 2007; Rosenbaum et al., 2010). However, this increase in versatility occurs at lower electron transfer efficiencies from the substrate to the electrode (i.e., coulombic efficiency). Since *S. oneidensis* was found in the

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anaerobic community of undefined mixed-culture MFCs (Kim et al., 2006), while lactate is not a common primary MFC substrate, we believe that *S. oneidensis* may play an important role as a terminal link between fermentation processes and electrode respiration in the anaerobic food-web of a BES anode compartment. To improve the community's conversion efficiency for a specific organic substrate into electric power, we need to understand how microbes interact in the mixed culture anodic food web. Only few recent studies have investigated the interactions of microorganisms in defined co-cultures in bioelectrochemical systems. Ren et al. (2007) studied the cellulose fermenter *Clostridium cellulolyticum* with the direct electron-transfer bacterium *G. sulfurreducens* to generate an electric current with cellulose as substrate, whereby the co-culture showed similar electrochemical performances as a pure-culture of *G. sulfurreducens* when fed with acetate. Read et al. (2010) studied biofilm formation and electrochemical performance of the Gram$^-$ ARBs *S. oneidensis*, *G. sulfurreducens*, and *P. aeruginosa* in combination with two Gram$^+$ fermenters: *Clostridium acetobutylicum* and *Enterococcus faecium*. They found an increased current production for all the tested binary co-cultures with *E. faecium*, while the co-cultures with *C. acetobutylicum* produced lower currents compared to the Gram$^-$ pure-cultures alone. It seems therefore, that the interactions between fermenters and ARBs can be various: neutral – with only a food-chain relationship (*G. sulfurreducens* and *C. cellulolyticum*); positive – with enhanced current production of the co-culture (all tested ARBs and *E. faecium*); and negative – with reduced current production of the co-culture (all tested ARB and *C. acetobutylicum*) compared to the ARB pure-culture. At this time point, a prediction of the type of interaction between a fermenter and an ARB is not possible.

Here, we studied how the physiology of *S. oneidensis* is influenced when it is grown in a co-culture with an unmetabolizable substrate, such as glucose. We combined *S. oneidensis* MR-1 with the homolactic fermenter *Lactococcus lactis* in a continuous-flow BES. *L. lactis* ferments C-6 sugars into L-lactate, and therefore a combination of both organisms should allow *S. oneidensis* to produce electric current with glucose as the primary fuel. Besides investigating the electrochemical behavior of the pure- versus the co-culture, we performed chemical, biochemical, and microscopy analyses, and at the end of the electrochemical experiment we recovered mRNA from the pure- and co-culture biofilms. The mRNA was used for a *S. oneidensis* transcriptional analysis with Affymetrix GeneChips. This analysis enabled us to ascertain if *S. oneidensis* MR-1 undergoes physiological changes in a synergistic co-culture fed with glucose.

2. Methods

2.1. Strains and media for bioelectrochemical systems

S. oneidensis MR-1 (a gift from Tim Gardner, Boston University, Boston, MA, USA) was grown in LB medium for strain maintenance. *L. lactis* LM 0230 (Efstathiou and McKay, 1977) was obtained from the Bioenergy Research Unit of the ARS, USDA, Peoria, IL. *L. lactis* was grown in M17 lactic acid bacteria medium (DSMZ medium 449) and 5 g/L sterile-filtered glucose was added after autoclaving. The defined anode medium for bioelectrochemical experiments was prepared according to Myers and Nealson (1988) and was modified by adding 1.27 mM K_2HPO_4, 0.73 mM KH_2PO_4, 125 mM NaCl, 5 mM HEPES, 0.5 g/L yeast extract, 0.5 g/L tryptone, and 5 g/L sodium β-glycerophosphate – a medium requirement of *L. lactis* (no addition of amino acids). After autoclaving, sterile filtered 1 g/L glucose, 20 mM sodium L-lactate, and 20 mM K_2HPO_4 were added. Analytical chemicals were ACS grade.

2.2. Reactor set-up

Two identical H-type electrochemical reactors were made of glass with an anode and cathode liquid chamber volume of 220 mL each (Supplementary Fig. S1). The anode and cathode chambers were separated by an anion exchange membrane (19.6 cm^2, AMI-7001 Membranes International, Glen Rock, NJ, USA). Each anode chamber was temperature controlled at 30 °C with a water jacket, stirred, continuously fed with defined medium at a hydraulic retention time (HRT) of 5–10 h, and was equipped with a carbon paper electrode (50 cm^2 geometric surface area, AvCarb P50, The Fuel Cell Store, San Diego, CA, USA), which served as working electrode. It was bound to a graphite rod (Poco Graphite Inc., Decatur, TX, USA) with carbon cement (CCC Carbon Adhesive, EMS, Hatfield, PA, USA). We used an Ag/AgCl (saturated KCl) reference electrode to control the working electrode (anode) potential. The cathode chambers (=counter electrode chamber) were operated in batch with a graphite block electrode ($3 \times 9 \times 1$ cm, PocoGraphite, Decatur, TX, USA). The entire assembled setup, including two 10-L feeding tanks, was autoclaved before the experiment. The tanks were used consecutively, whereby one tank fed both anode chambers simultaneously during the *S. oneidensis* pure-culture stages. At all time, the medium tanks and the reactors were kept anaerobic by keeping it under a positively pressured 20% CO_2/80% N_2 atmosphere.

2.3. Electrochemical measurements

Steady-state *S. oneidensis* biofilms were grown with lactate as electron donor at a working electrode under potentiostatic conditions at 0.4 V (all potentials refer to standard hydrogen electrode, SHE) (VSP potentiostat, BioLogic, Knoxville, TN, USA, in a three electrode setup) in two parallel reactor setups. Before inoculation with an overnight culture of *S. oneidensis*, blank electrochemical measurements were conducted in the growth medium. In some runs, complex media components that were required for the growth of *L. lactis* (yeast extract, tryptone, β-glycerophosphate) were added to a growing *S. oneidensis* culture one at a time to evaluate the bioelectrochemical effects of those components (after we verified that those media components had no effect, they were added to the medium from the start). Once stable bioelectrochemical performance with *S. oneidensis* was achieved, one reactor was switched to a medium tank without L-lactate (from now on called *reactor SL*, as opposed to *reactor S* which still was fed with 20 mM lactate), while 1 g/L glucose was fed to both reactors. When residual lactate was depleted, 3 mL overnight grown *L. lactis* was inoculated into reactor SL. Cyclic voltammetry tests (−0.3 to 0.7 V, $v = 1$ mV/s) were performed regularly (every 48 h) during the entire experimental run. This experimental series was repeated several times with similar performance but with different length of operating periods. From two of these trials, biofilm RNA was collected for transcriptional analysis. The purity of the culture was confirmed by plating of the reactor liquid at the end of each experiment (*S. oneidensis* forms characteristic pinkish colonies).

2.4. Chemical analysis

Anode effluent samples were taken every other day to determine HRT, effluent pH, L-lactate (Accutrend Lactate Analyzer, Roche Diagnostics), and other soluble metabolites. Filtered samples (0.2-μm nitrocellulose filter, Millipore, Billerica, MA, USA) were analyzed for sugars, organic acids, and ethanol using a SpectraSYSTEM liquid chromatography system equipped with a refractive index detector (Thermo-Fisher Scientific Inc.) and with an organic acids column (Aminex HPX-87H Column, Bio-Rad

Laboratories Inc., Hercules, CA, USA). Samples were run at 65 °C and eluted at 0.6 mL/min with 5 mM sulfuric acid.

2.5. SEM imaging

At the end of the experiment a part of the carbon paper electrode was sampled for SEM imaging: the electrode samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, followed by a serial ethanolic dehydration, critical point drying, and thin gold coating. Images were taken with a Hitachi S-450 scanning electron microscope at 20 kV accelerating voltage.

2.6. Microarray analysis

2.6.1. Chemicals and reagents used for microarrays

All general chemicals for molecular biological work were certified RNase/DNase free. Qiagen Inc. (Valencia, CA) supplied the RNA protect reagent and the DNA purification kit. Other specific reagents and chemicals used during isolation/purification of RNA and during various steps of *Shewanella* chips hybridization (Affymetrix Inc., Santa Clara, CA) were purchased form several different vendors: Superscript II reverse transcriptase, DTT, random hexamers, and BSA from Invitrogen Inc. (Carlsbad, CA); GeneChip labeling reagent, One-phor-all buffer, and B2 oligo from Affymetrix Inc.; DNAse from Pierce Biochemicals Inc. (Rockford, IL); MES stock, lysozyme, Goat IgG, and 200 proof ethanol from Sigma–Aldrich (St. Louis, MO); Terminal transferase, Herring sperm DNA, and dNTPs from Promega Inc. (Madison, WI); Biotinylated Anti-Streptavidin antibody from Vector laboratories (Burlingame, CA); SSPE, Streptavidin, SAPE, 10% Tween 20, NaOH, and HCl from ThermoFisher Scientific (Waltham, MA); and RNAse-free DNAse I enzyme for RNA purification, TE Buffer (pH 8.0), Superase 1n, 5 M NaCl, and nuclease-free water were from Ambion (Austin, TX).

2.6.2. RNA sampling and isolation

RNA for microarray analysis was sampled from two S and two SL bioelectrochemical reactors. The carbon paper electrodes were removed from the reactor, bathed in Qiagen RNA-protect for 30 s and immediately frozen at −80 °C. The biofilm samples were loosened, but not removed, from the electrode backbone by scraping with a sterile razor blade. The electrode backbone was then washed with 2 mL RNA protect and the biofilm-carbon sludge was transferred to a 15 mL tube. Seven millilitres ice-cold phosphate buffer saline (PBS) was added, the mix was vortexed on highest speed and centrifuged for 10 min at 5500*g*. The supernatant was decanted and replaced with 7 mL ice-cold PBS. Then, the mix was sonicated at 7 W for 30 s on ice. Vortexing, centrifuging, and sonicating was repeated twice. Then, the pellets were resuspended in 0.75 mL NAES buffer (50 mM sodium acetate buffer, 10 mM EDTA, and 1% SDS at pH 5). RNA was isolated with a phenol:chloroform extraction protocol similar to Cury and Koo (2007). The isolated RNA was purified from genomic DNA contaminations with Ambion DNase I treatment following the manufacturer's instructions. RNA yields were quantified with a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE) and UV 260/280 ratios were calculated to check purity of each RNA sample. RNA quality was verified in a 1.5% agarose electrophoreses gel with ethidium bromide staining.

2.6.3. Microarray hybridization

A previously described protocol (Driscoll, 2008; Faith et al., 2007) was used for microarrays on *S. oneidensis* chips from Affymetrix Inc. In brief, approximately 10 μg of each RNA sample was used for cDNA synthesis through reverse transcription, cDNA purification, and cDNA-fragmentation. This was followed by labeling of cDNA and 16 h of hybridization at 45 °C on *S. oneidensis* arrays. The labeled arrays were subjected to several cycles of washing and staining using Affymetrix Wash buffers A and B, Goat IgG, Streptavidin, Anti-streptavidin, and SAPE according to the Affymetrix protocol for prokaryotic arrays. This was followed by scanning of the stained arrays with Affymetrix GeneChip Scanner Model 3000. The *S. oneidensis* MR-1 microarray data have been submitted to Gene Expression Omnibus (accession number GSE20343).

2.6.4. Statistical analysis

Microarray data were analyzed with the lemma (Laplace approximated EM Microarray Analysis) package, available from http://www.cran.r-project.org/ by Bar and Schifano. The software is based on the statistical model proposed by Bar et al. (in press). To account for the large number (3949) of hypotheses (i.e., genes tested), we used the Benjamini–Hochberg adjustment to the *p*-values, which allows to control the false discovery rate (*fdr*) at any desired level (Benjamini and Hochberg, 1995). Visualization of the results as heatmaps was performed in JMP 8.0 (SAS Institute Inc.).

3. Results and discussion

3.1. BES performance of a S. oneidensis pure- and co-culture

S. oneidensis was grown on carbon paper electrodes in two, simultaneously-operated, continuously-fed BES reactors under potentiostatic control at 0.4 V (Fig. S1). One of the two reactors (SL) was transitioned to co-culture operation once stable performance of *S. oneidensis* was observed. Within a few hours after switching from a lactate-fed *S. oneidensis* pure-culture to a glucose-fed co-culture with *L. lactis*, the current generation recovered to the original value of the pure-culture. We performed four different trials and report final steady-state current densities and the steady-state coulombic efficiencies (Table 1). The cyclic voltammogram in Fig. 1 shows the catalytic current wave of the *S. oneidensis* pure-culture in trial 2 at different time points of the experiment with an onset potential of ∼+0.1 V. The current production and coulombic efficiencies within each trial were very similar for the two reactors, but varied between the individual trials (we commonly observed broad variations in *S. oneidensis* performance between trials although the experimental conditions were unaltered). Therefore, we performed paired, two-tailed *t*-tests to evaluate the experimental differences between *S. oneidensis* in pure-culture and co-culture, and found no statistical difference between the current production (p-value = 0.709) and coulombic efficiency (p-value = 0.804) of reactor S versus reactor SL. Thus, from an electrochemical standpoint, *S. oneidensis* showed the same performance in pure-culture with lactate feeding and in co-culture with glucose feeding.

We operated each trial for weeks with a continuous flow of nutrients (i.e., in a chemostat) and added sufficient phosphate buffer (up to 100 mM) to our medium to prevent a pH value lower than 5.8 (especially during the *L. lactis* acid formation phase it is important to maintain a high enough pH for sufficient *S. oneidensis* activity). The pH levels and metabolic regime during the transition from a pure-culture to a co-culture in reactor SL during experimental trial 2 is given (Fig. 2). The feeding concentration of glucose was adjusted from initially 1 g/L at the starting time of glucose feeding to achieve excess lactate concentrations in the effluent (to prevent substrate limitation effects). Acetate was mainly formed as a by-product during lactate oxidation by *S. oneidensis*, but *L. lactis* can also produce small amounts of acetate as a by-product of glucose fermentation. Even though *L. lactis* is considered a homolactic fermenting organism, the growth and substrate conditions define how complete the homo-fermentative reaction is. Other products typical of a mixed acid fermentation (e.g., pyruvate, acetate,

Table 1
Current density and coulombic efficiency (CE) of a *S. oneidensis* pure-culture (S) and a co-culture with *L. lactis* (SL).

Trial	Duration (days)[a]	Current density (μA/cm^{-2})		CE[b] (%)	
		(S)	(SL)	(S)	(SL)
1	13	22.8	19.8	20.3	13.7
2	30	22.5	21.5	17.9	16.7
3	32	6.8	6.4	1.3	1.4
4	32	11.34	19.9	4.8	9.9
Average	26.75	15.9	16.9	11.1	10.4
t-Test (paired, two tailed) – *p*-value:		0.709		0.804	

[a] The significantly shorter experimental duration of trial 1 is due to starting this trial with the final media composition containing all complex components, while in the other trials complex media components (yeast extract, tryptone) were added one by one during the experiment to exclude influences to the electrochemical results.
[b] The coulombic efficiency CE represents the ratio of the current (as C/s) and the charge resulting from lactate oxidation as follows: $CE = I/[z \times F \times fr \times c_{Lactate}]$ with I—current in C/s, z—number of electrons transferred per molecule, F—Faraday's constant, fr—reactor flow rate in L/s, and $c_{Lactate}$—molar concentration of consumed lactate for S, and for SL ([glucose in] – [glucose out]) × 2 – [lactate out].

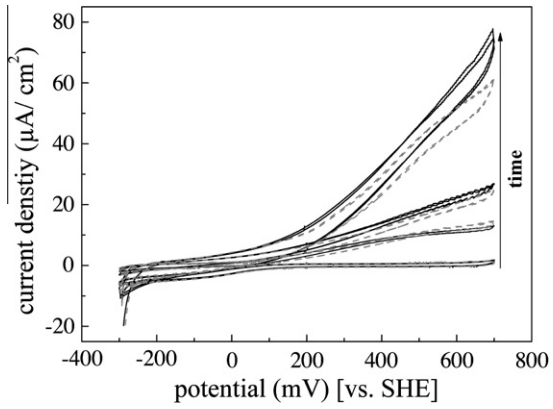

Fig. 1. Cyclic voltammetry plots of a *S. oneidensis* pure-culture from different time points (days 1, 7, 14, and 28) during the continuous BES reactor operation of trial 2. Bold black lines represent reactor S with a *S. oneidensis* pure-culture, dashed gray lines represent reactor SL that was operated with a *S. oneidensis*/*L. lactis* co-culture and glucose substrate from day 23 on (before that time point it contained only *S. oneidensis* and lactate substrate similar to reactor S). Potentials were scanned at 1 mV/s.

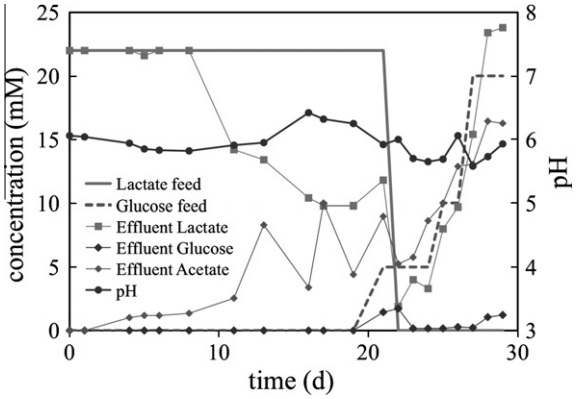

Fig. 2. Chemical analyses of metabolites (left axis) and pH (right axis) during a continuous-flow co-culture BES experiment with *S. oneidensis* MR-1. *L. lactis* was inoculated on day 22 after the medium was switched from lactate to glucose as carbon source.

ethanol) may compose up to 10% of the fermentation products (Cocaign-Bousquet et al., 1996). Many microbial fermentation reactions can be enhanced by specific product removal to overcome product toxicity effects or feed-back inhibition (Ezeji et al., 2007). In our study, where *S. oneidensis* removed lactate formed during glucose fermentation by *L. lactis*, we did not see such shift in fermentation patterns because *L. lactis* converts glucose almost stoichiometrically into lactate. In addition, at our low glucose concentrations (1–4 g/L) no toxicity of lactate was observed.

3.2. L. lactis alone showed no significant electrochemical activity

Despite recent reports on electric current generation by *L. lactis* (Freguia et al., 2009; Masuda et al., 2010), our results showed no significant current generation by a *L. lactis* pure-culture. We measured inconsequently low electrochemical activity for a pure-culture of *L. lactis* in defined (minimal) medium (~0.4 μA/cm^2; note that we report up to 20 μA/cm^2 for a pure-culture of *S. oneidensis*). Freguia et al. (2009), who also worked with a minimal medium for pure-culture growth of *L. lactis*, did not report current densities/surface area, but we estimated that 2.5 μA/cm^2 was the upper limit for their study (based on the given geometric surface area of the utilized three-dimensional graphite felt). These authors reported that a total of 1% of the electrons from glucose were redirected from lactate production to pyruvate, acetate, and electric current, with a very low coulombic efficiency of 0.275% for the current generation. With the knowledge that fermentation side products are well known for *L. lactis* (Cocaign-Bousquet et al., 1996) and compared to the much higher current production of *S. oneidensis*, we concluded that *L. lactis* cannot generate significant electric current in absence of externally-supplied redox shuttles. In rich medium (#449, DSMZ, Germany, containing 15 g peptones and 2.5 g yeast extract), we measured 7 μA/cm^2 for a *L. lactis* pure-culture with a much higher abiotic background current (20 μA/cm^2 compared to 0.2 μA/cm^2 background current in the defined medium with 0.5 g/L each yeast extract and tryptone). This indicates that media redox components can serve as electron shuttles for *L. lactis* and, indeed, Masuda et al. (2010) reported current production by *L. lactis* in the presence of flavin-type redox-mediators contained in yeast extract (the electrode surface related current density could not be derived from the publication). But since the influence of *L. lactis* on the *S. oneidensis* bioelectrochemical physiology was the target of this study, all experiments were performed in defined medium with low amounts of complex medium components.

3.3. Continuous co-cultures were dominated by S. oneidensis-direct electron transfer processes

Although *S. oneidensis* can produce flavin-type mediators to enhance electron transfer to the electrode (Marsili et al., 2008; von Canstein et al., 2008), our cyclic voltammetry analyses in *S. oneidensis* pure-culture experiments did not indicate the involvement of flavin-type mediators (Fig. 1), which, if they had been present, could have enabled *L. lactis* to participate in current generation. In medium of similar composition to ours, at a slightly

more basic pH (pH 7 instead of ~6.2) Marsili et al. (2008) found the (ribo)flavin mid-peak potential (and corresponding onset in the catalytic current curve) to be ~−0.2 V. Malinauskas (2008) determined the mid-peak potential of riboflavin on graphite electrodes to be −0.22 V. Our voltammograms (Fig. 1) show the steady increase of a catalytic current with increasing activity of the culture at an onset potential of ~0 to +0.1 V, which indicates a different catalytic component than the flavins, such as an outer membrane *c*-type cytochrome, that exhibits direct electron transfer to the electrode. Meitl et al. (2009) determined the standard potentials for *S. oneidensis* outer membrane cytochromes (MtrC and OmcA) to be 0.0–0.1 V vs. SHE, which is very close to the onset potential found in our study. In another study, Cho and Ellington (2007) used anaerobically-grown, washed cells of *S. oneidensis* in a phosphate buffer electrolyte to measure non-turnover cyclic voltammograms (i.e., no catalytic wave occurs, since no substrate is oxidized during the test) and found a redox system with a mid-peak potential of ~0.0 V vs. SHE. Since those cells were washed before the cyclic voltammetry tests, the observed redox system likely does not result from soluble mediators, but instead from membrane bound redox components, such as *c*-type cytochromes. Electron microscopy analysis of the electrodes from reactors *S* and SL showed an open monolayer of *S. oneidensis* cells, instead of a complex biofilm, regardless of the presence of *L. lactis* (Fig. S2). The latter microbe grew almost exclusively in a planktonic state at high densities with only some cells (cocci) attached on the outside of the monolayer of *S. oneidensis* for reactors SL (Fig. S2). The combination of a thin and open biofilm structure of primarily *S. oneidensis* for the co-culture experiments with continuous-flow reactor operating conditions at short hydraulic retention times of 5–7 h, assured an electron-transfer mechanism that was dominated by direct electron transfer. Our data shows that a mediated electron-transfer mechanism was not important, likely because the soluble redox shuttles were washed out and never reached high enough concentrations. This not only explains why we did not measure mediator activity, but also eliminated redox mediators from *S. oneidensis* that possibly could have been used by *L. lactis* to respire with the anode. Therefore, our experimental design is pertinent for BESs that are dominated by the direct-electron-transfer mechanism of exoelectron transfer to the anode.

3.4. Gene expression of S. oneidensis in pure- and in co-culture is indifferent

To evaluate not only the experimental changes, but also molecular physiological changes, we performed gene expression analysis on 3949 *S. oneidensis* genes with Affymetrix gene chips (after data normalization and exclusion of chip miss-reads from a total of 4077 *S. oneidensis* gene probes on the chip) for the *S. oneidensis* pure-culture (S; $n = 2$) and co-culture electrode biofilms (SL; $n = 2$) from the BES electrodes (for trials 1 and 2). The statistical analysis was performed using the R package lemma (Laplace approximated EM Microarray Analysis), and no statistically significant differences in expression between pure- or co-culture were detected. This result is in accordance with our electrochemical and metabolic data: the presence of *L. lactis* as a metabolic partner organism had no significant effect on the physiological state or activity of *S. oneidensis*. Supporting our statistical conclusions, a heatmap visualizes only slight differences in the color pattern between reactor S and SL (Fig. 3). The similarity becomes even more clear when the expression levels of S and SL are averaged (column S + SL), or when the difference is shown (column S − SL). The latter heatmap should cancel out the expression levels of individual genes if they are similarly expressed in both samples. The fact that only colors in the immediate range around the general mean gene expression level of the genome (mean{$\log_2$[signal intensity]} = 8.56) are shown in this column, confirms visually that *S. oneidensis* gene expression is statistically not different for both samples.

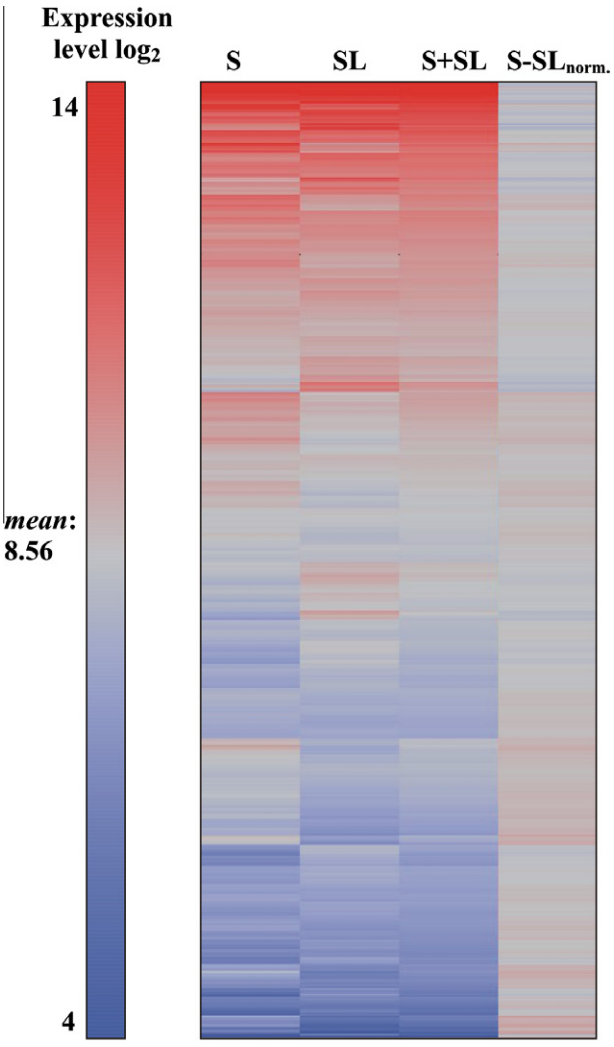

Fig. 3. Heatmap showing the clustered gene expression levels of *S. oneidensis* in pure-culture (column S, $n = 2$), co-culture (column SL, $n = 2$), as the mean of pure- and co-culture (column S + SL, $n = 4$), and as the normalized difference of the two culture tests (column [S − SL], normalized by the mean genomic expression, $n = 4$). Individual genes in all columns are ordered by the mean expression level of S + SL. The expression level represents the $\log_2$ of the Affymetrix GeneChip fluorescence signal intensity and is color-coded following the scheme on the left. The mean of the gene specific mean-square-errors (m_g) of the statistical expression analysis is mean [m_g] = 0.51. The smallest *p*-value was greater than 0.3, yielding no discoveries at the selected 5% *fdr* level. The *p*-values are obtained by fitting a parametric model to the data.

3.5. S. oneidensis and L. lactis establish a neutral food-web relationship in BES

Here, we showed that the performance of *S. oneidensis* in a BES under controlled experimental conditions (lactate feeding) and in a specific microbial food-web (with no food competitors being present) was comparable. This suggests the possibility of designing microbial communities to exploit specific food webs for enhanced bioelectrochemical conversion of a wide spectrum of organic waste products. For instance, while *S. oneidensis* or *L. lactis* alone cannot generate electric current from glucose, they can do so in a co-culture. We found that the *S. oneidensis* biofilm structure (i.e., electrode coverage) and physiological state (i.e., gene expression)

were the same for the pure-culture and the co-culture. The fermentation reaction of *L. lactis* occurred in planktonic state and did not influence the bioelectrochemical reaction of *S. oneidensis*, besides the provision of lactate as the electron donor (i.e., a neutral relationship). Thus, the interaction between the two organisms occurred only at the substrate level. Our work supports the hypothesis that fermenting bacteria only provide intermediate products to ARB, such as from the genera *Shewanella* and *Geobacter*, in BESs that are dominated by direct electron transfer. Under such conditions when external redox shuttles are absent, the very small electric currents generated by fermenters are inconsequential. This finding is in agreement with the results of Ren et al. (2007) for a co-culture of *G. sulfurreducens* and *C. cellulolyticum*, but not with the study of Read et al. (2010), who found a negative impact of the fermenter *C. acetobutylicum* and a positive, synergistic effect of the fermenter *E. faecium* on the current production by *S. oneidensis*, *G. sulfurreducens*, and *P. aeruginosa*. It is, thus, clear that the interactions between ARB and fermenters cannot be simply predicted, especially since some ARB only perform direct electron transfer, some only interact with the electrode through redox mediators, and some are capable of both. This opens many possible ways of interaction between the ARB and fermenters. While substrate level interactions between microorganisms in a mixed-culture BES are important to uncover (such as in our study), the identification and investigation of true synergistic fermenter–ARB relationships will be much more essential for the improvement of BES performance.

4. Conclusion

The understanding of metabolic network relationships in mixed-culture BES, which can be of neutral, positively enhancing, or negatively impeding kind, becomes an important step in the advancement of BES development. Here, we studied the relationship between the homolactic fermenter *L. lactis*, which converts glucose to lactate, and the electricigen *S. oneidensis*, which converts lactate into electric current at conditions that are pertinent to conventional BES operation. With electrochemical, metabolic, and gene expression analysis we determined that the two cultures establish a pure food-based relationship, because the physiology and electrochemical activity of *S. oneidensis* was similar regardless of the presence of *L. lactis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.10.033.

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