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ORIGINAL PAPER

Probing the redox metabolism in the strictly anaerobic, extremely thermophilic, hydrogen-producing *Caldicellulosiruptor saccharolyticus* using amperometry

Natalie Kostesha · Karin Willquist · Jenny Emneus · Ed W. J. van Niel

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Abstract Changes in the redox metabolism in the anaerobic, extremely thermophilic, hydrogen-forming bacterium *Caldicellulosiruptor saccharolyticus* were probed for the first time in vivo using mediated amperometry with ferricyanide as a thermotolerant external mediator. Clear differences in the intracellular electron flow were observed when cells were supplied with different carbon sources. A higher electrochemical response was detected when cells were supplied with xylose than with sucrose or glucose. Moreover, using the mediated electrochemical method, it was possible to detect differences in the electron flow between cells harvested in the exponential and stationary growth phases. The electron flow of *C. saccharolyticus* was dependent on the NADH- and reduced ferredoxin generation flux and the competitive behavior of cytosolic and membrane-associated oxidoreductases. Sodium oxamate was used to inhibit the NADH-dependent lactate dehydrogenase, upon which more NADH was directed to membrane-associated enzymes for ferricyanide reduction, leading to a higher electrochemical signal. The method is noninvasive and the results presented here demonstrate that this method can be used to accurately detect changes in the intracellular electron flow and to probe redox enzyme properties of a strictly anaerobic thermophile in vivo.

Keywords *Caldicellulosiruptor saccharolyticus* · Lactate regulation · Anaerobiosis · Electrochemistry · Mediated amperometry · Ferricyanide

Introduction

Redox reactions are quintessential to maintain life. The overall cellular redox environment consists of different redox couples, such as the $NAD(P)^+$/NAD(P)H, the cysteine/cystine couple, the glutathione/glutathione disulfide couple, and the reduced ferredoxin (Fd_{red}) and oxidized ferredoxin (Fd_{ox}) couple (Angenent et al. 2004; Brown et al. 1998; Buchanan et al. 2002; Schafer and Buettner 2001). It has been established that the redox environment is closely related to the concentration of the redox couples, which regulates different cellular functions such as energy metabolism, the biosynthesis of macromolecules, signal transduction, and the cell life cycle by modulating enzyme activity and transcription (Arrigo 1999; Nakamura et al. 1997; Powis et al. 1995; Williamson et al. 1967).

It is necessary to gain insight into the redox metabolism of the cell to understand its physiology, but this is a difficult task as the cellular redox environment includes all redox couples participating in the metabolic network. Traditional in vitro techniques for the determination of the redox status have several drawbacks, including: (i) failure to probe the overall redox status of the cell, since only individual redox couples are determined (Schafer and Buettner 2001); (ii) requirement of cell lysis, which can influence the redox status (Almeida et al. 2008; Gao et al. 2006; Zhang et al. 2000); and (iii) lack of considering the association with the allosteric enzyme-cofactor, which is known to have a considerable influence on

N. Kostesha and K. Willquist contributed equally to the paper.

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N. Kostesha (✉) · J. Emneus
Department of Micro- and Nanotechnology,
Technical University of Denmark, Ørsteds Plads 345 East,
2800 Kgs. Lyngby, Denmark
e-mail: natalie_kostesha@nanotech.dtu.dk

K. Willquist · Ed W. J. van Niel
Department of Applied Microbiology, Lund University,
P.O. Box 124, 221 00 Lund, Sweden

the in vivo redox status. Standard techniques currently used to probe the changes in the redox couples of disrupted cells are spectrophotometry (Almeida et al. 2008; Zhang et al. 2000), fluorometric cytometry (Krylov et al. 1999) and mass spectrometry (Mashego et al. 2007). Until now, probing the redox state of intact cells has presented a challenge.

However, electrochemical methods have enabled measurements of the intracellular redox state in vivo, thereby obtaining information on the cellular redox state without cell disruption, its dynamics, and the kinetics of redox reactions in vivo (Catterall et al. 2001; Ikeda 2004; Ikeda and Kano 2001; Takayama et al. 1993). For instance, the sugar metabolism in *Saccharomyces cerevisiae* could successfully be monitored by these methods (Heiskanen et al. 2009; Kostesha et al. 2009b; Spegel et al. 2007).

This paper describes the development of a method of studying the redox metabolism of strictly anaerobic and thermophilic bacteria based on flow injection analysis (FIA) combined with ferricyanide-mediated amperometry. Thermophilic anaerobes have recently attracted considerable scientific and industrial interest. *C. saccharolyticus* was chosen as the model organism for several reasons, such as its ability to produce almost theoretical amounts of hydrogen from plant biomass (de Vrije et al. 2009), its ability to grow on various carbohydrates ranging from monomers (pentose and hexoses) (Rainey et al. 1994; Van Fossen et al. 2009) to complex hemicellulosic polymers (de Vrije et al. 2009), and to co-metabolize xylose and glucose (van de Werken et al. 2008; Van Fossen et al. 2009), rendering it interesting for the fuel and chemical industry. However, recent studies have shown that its metabolism is partly directed toward lactate production in the transition to the stationary phase, which has a negative effect on the hydrogen yield (Willquist and van Niel 2010). The hydrogen yield is also affected by the concentration of dissolved hydrogen in its surroundings (Kraemer and Bagley 2007; van Niel et al. 2003). A high concentration inhibits the hydrogen-generating hydrogenase reaction, leading to an increase in the NADH/NAD ratio and a metabolic shift toward the production of reduced fermentation products such as lactate (van Niel et al. 2003). Moreover, recent results have shown that different carbon sources induce different levels of lactate formation: less lactate being formed on a xylose substrate than on glucose or sucrose substrates (Willquist 2010). The electrochemical assay was developed as a means to explain these observations.

Theory behind the electrochemical method

The working principle of the amperometric assay is shown in Fig. 1. Overall, the electrochemical signal illustrates the intracellular electron flow, which will depend on the sugar uptake rate, the sugar oxidation rate, and the flux distribution at the pyruvate node. The redox reactions involved in the oxidation of the substrate are monitored by an amperometric assay, where membrane-associated enzymes transfer the electron from the reduced cofactors NADH and Fd_{red} to ferricyanide (FC), reducing it to ferrocyanide (FoC), which in turn transfers its electrons to an anode generating an amperometric signal. When the sample is injected into the continuously supplied buffer, the FoC will immediately be oxidized on the electrode surface, forming FC. The oxidation of FoC acts as a single-electron transfer mechanism and the resulting current is seen as a peak, denoting the direct electron transfer (Fig. 1). FC has several advantages as a mediator. Firstly, it has a high formal potential ($E^{\circ\prime} = 274$ mV vs. Ag/AgCl; Kolthoff and Lingane 1952). Since electrons flow from donors with low formal potential [Fd_{red} ($E^{\circ\prime}$ approx -400 mV; Angenent et al. 2004)] via the oxidation of NADH ($E^{\circ\prime} = -320$ mV; Angenent et al. 2004) to acceptors with higher formal potential [FC ($E^{\circ\prime} = 274$ mV vs. Ag/AgCl; Kolthoff and Lingane 1952)], the reactions that couple the reduction/oxidation reactions occur spontaneously ($\Delta G < 0$). Secondly, NADH and Fd_{red}-dependent oxidoreductases located on the plasma membrane have generally a high affinity for FC, and can thus stimulate rapid electron transfer (Kondo and Ikeda 1999; Shaw et al. 2008; Walsh et al. 1983).

The signal is the result of the oxidation of the carbon sources in the Embden–Meyerhof–Parnas pathway (EMP) (de Vrije et al. 2007), which generates NADH, and the oxidation of pyruvate to acetate, which generates Fd_{red} (Fig. 2). Glucose, sucrose, and xylose are transported into the cell by ATP-binding cassette (ABC) transporters (van de Werken et al. 2008). Intracellular glucose enters the pathway directly, while intracellular sucrose is hydrolyzed by invertases yielding an equimolar mixture of glucose 6-phosphate and fructose 1-phosphate. Intracellular xylose is hydrolyzed to fructose 6-phosphate and glyceraldehyde 3-phosphate, which are subsequently metabolized via the Embden–Meyerhof pathway (van de Werken et al. 2008). At the pyruvate node, pyruvate can either be oxidized to acetate, which contributes to the reduction of one ferredoxin molecule, or it can be reduced to lactate or ethanol at the expense of one NADH molecule or two NADPH molecules, respectively (Fig. 2; van de Werken et al. 2008). However, ethanol has only been found in trace amounts in the culture supernatant (de Vrije et al. 2007; Willquist and van Niel 2010) and is therefore not considered in this study. Consequently, if pyruvate is oxidized to acetate and CO_2, two Fd_{red} and two NADH molecules, which can both contribute to the amperometric response, are produced from glucose. On the other hand, reduction to lactate would remove any reduced cofactor and hence

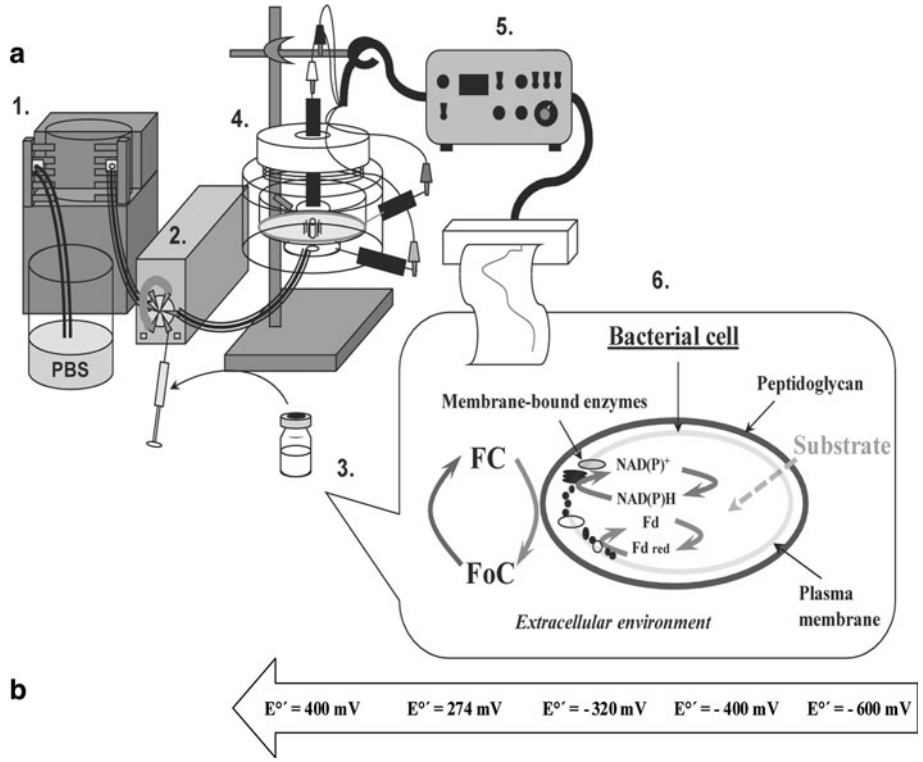

Fig. 1 **a** Illustration of the FIA setup. FIA was performed at a fixed potential of 400 mV. Using a peristaltic pump (*1*). PBS was continuously pumped via the six-port injection valve (*2*) into the electrochemical cell (*3*), where three electrodes (an Ag/AgCl saturated reference electrode, a Pt counter electrode and a Pt working electrode) were connected to a potentiostat (*4*). Signals were recorded using a pen recorder (*5*). Supernatant from centrifuged samples (*6*) were loaded using the six-port injection valve. The callout shows the schematic conversion of ferricyanide (*FC*) to ferrocyanide (*FoC*) by membrane-bound redox enzymes located on the plasma membrane of *C. saccharolyticus* cells. FC and FoC are the oxidized and reduced forms of the mediator, Fd_{red} reduced ferredoxin, *Fd* ferredoxin, respectively. **b** A schematic representation of whole cell-mediated bioelectrocatalysis. Energy rich electrons originate from a substrate (glucose), which is metabolized through cellular metabolism (using the EMP pathway) generating reduced Fd_{red}, and NADH, which are oxidized by the membrane-bound enzymes that reduce FC. FC enhances electron transfer to the electrode surface. The electron transport proceeds along a potential gradient controlled by the formal potentials ($E^{o\prime}$) of the redox components and the electrode potential (E_{app})

compete with the reduction of the mediator for NADH oxidation, resulting in a lower amperometric signal.

Materials and methods

Reagents and buffers

Potassium ferricyanide (FC; $K_3[Fe(CN)_6]$), glucose, xylose, sucrose, sodium oxamate, NADH, menadione, and the Bradford reagent were obtained from Sigma (St. Louis, MO, USA). Potassium ferrocyanide (FoC; $K_4[Fe(CN)_6]$) was purchased from Fluka BioChemica (Buchs, Germany). All chemicals were of analytical grade and were used without further purification. All solutions were prepared using Milli-Q water (Millipore, Bedford, MA, USA).

Phosphate saline buffer (PBS) was used during incubation and for the flow injection analysis. The PBS contained 2 mM KH_2PO_4, 10 mM $Na_2HPO_4{\cdot}2H_2O$, 2.7 mM KCl, and 137 mM NaCl, and was adjusted to pH 7.0 with KOH. Solutions of glucose, sucrose, and xylose were prepared in concentration of 100 g/L in PBS, and ferricyanide (FC; 500 mM in PBS) were freshly prepared and filtered (Millex-GS 0.22 μm, Millipore, Co., Cork, Ireland). Sugar-containing solutions were prepared directly before use. The stock solution of FC was stored in a light-proof flask at 4°C. The stock solution of 10 mM sodium oxamate was prepared in PBS buffer immediately before use. All solutions were degassed with vacuum before the experiments, using the water jet vacuum pump, and then stored at room temperature.

Microorganism and medium

Caldicellulosiruptor saccharolyticus was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and stored in glycerol stocks at −80°C until use. The microorganism was cultivated in a DSM640 medium without trypticase (http://www.dsmz.de/microorganisms/html/media/medium000640.html). The carbon sources (10 g/L: glucose, sucrose, or

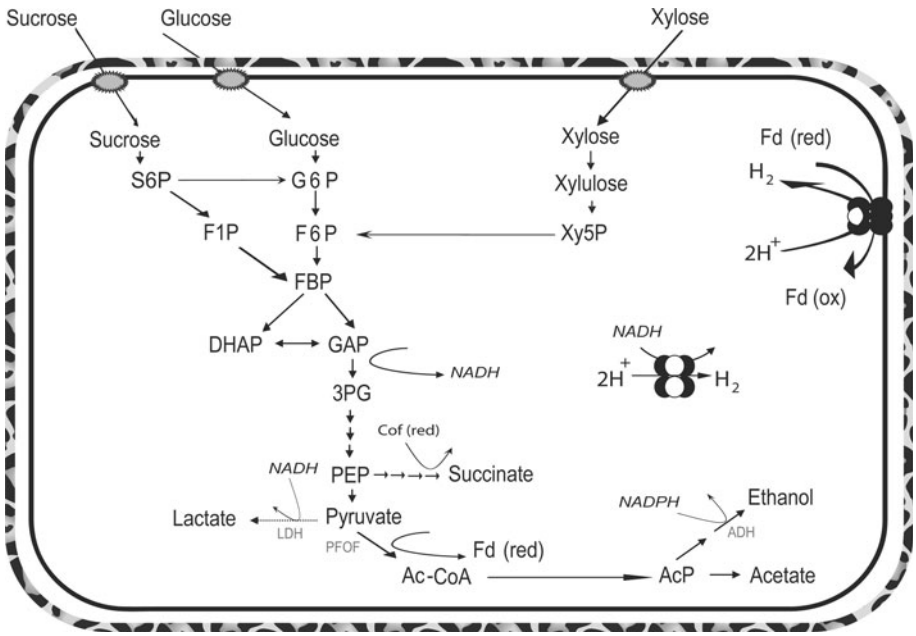

Fig. 2 Glucose, sucrose, and xylose metabolism in *Caldicellulosiruptor saccharolyticus.* Sucrose 6-phosphate (*S6P*); fructose 1-phosphate (*F1P*); glucose 6-phosphate (*G6P*); fructose 6-phosphate (*F6P*); xylulose 5-phosphate (*Xu5P*); fructose 1,6-bisphosphate (*FBP*); glyceraldehyde 3-phosphate (*GAP*); dihydroxyacetone phosphate (*DHAP*); 3-phosphoglycerate (*3PG*); phosphoenolpyruvate (*PEP*); acetyl-CoA (*AcCoA*); acetyl-phosphate (*AcP*), reduced ferredoxin (Fd_{red}); reduced cofactor (Cof_{red}); glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); lactate dehydrogenase (*LDH*); pyruvate ferredoxin oxidoreductase (*PFOR*), alcohol dehydrogenase (*ADH*)

xylose), $MgCl_2$ (0.4 g/L), and cysteine (1 g/L) were sterilized separately and added to autoclaved medium. The organisms were pre-cultured in a serum flask (250 mL) with a working volume of 50 mL in the medium described above, but with 4 g/L carbon source and 0.75 g/L cysteine.

Cultivation and harvesting conditions

C. saccharolyticus was cultivated under a nitrogen atmosphere (1 atm) in a 3-L Applikon bioreactor (Schiedam, The Netherlands), with a working volume of 1 L. The pH was monitored with an Applikon Biocontroller 1030 (Schiedam, The Netherlands) and maintained at 6.5 by the addition of 4 M NaOH. The temperature was maintained thermostatically at 71 ± 1°C, and the stirring rate was set to 250 rpm.

Cells were harvested under anaerobic conditions in the exponential (0.5 g/L) and stationary phases (1.4 g/L), and centrifuged under anaerobic conditions at 5,000 rpm for 5 min at 4°C using an Avanti J-25I centrifuge with a JA-10 rotor from Beckman Coulter Inc. (Fullerton, CA, USA). The cell dry weight (cdw) was determined as previously described by de Vrije et al. (2007), with the exception that 10–15 mL cell suspension was used and re-suspended in anaerobic PBS instead of double-distilled H_2O to avoid cell lysis (Bielen et al. 2010). The cells were then transferred to an anaerobic chamber (PlasmLab, Michigan, USA), containing a gas mixture consisting of: 85% N_2, 10% H_2, and 5% CO_2, where the cells were washed twice and re-suspended in anaerobic PBS to a final cell mass of 5 g/L.

Mediator-assisted assessment of the redox dynamics

Two different combinations of mediator were evaluated, i.e., (i) a combination of menadione (0.1 mM) and FC (20 mM) and ii) FC (20 mM) only. Cell suspensions (1 mL) were transferred to serum flasks (50 mL), to which 0.4 mL of mediator solution was added. Carbon sources, i.e., glucose, sucrose, or xylose, were added separately to each serum flask to a final sugar concentration of 10 g/L. Finally, PBS was added to adjust the final volume to 10 mL. The serum flasks were sealed to maintain anaerobic conditions and incubated at 70°C for 60 min. Samples (0.5 mL) were withdrawn every 10 min from each flask and centrifuged at 10,000 rpm for 60 s using a Biofuge A table centrifuge (Heraeus Christ, Osterode, Germany). The sample was immediately frozen in liquid nitrogen and stored at −20°C. Prior to analysis of the accumulated FoC, the samples were defrosted, vortexed (Fisher Scientific, Loughborough, UK), and diluted 1:40 with PBS.

Flow injection analysis

The working platinum electrode (a Pt disc with a diameter of 1.3 mm, Bioanalytical Systems Ltd, Kenilworth, UK) was polished with 0.25-μm diamond slurry and alumina

slurry (0.1-μm; Struers, Ballerup, Denmark) on a Microcloth (Buehler, Lake Bluff, IL, USA). After polishing, the electrode was ultrasonicated in Milli-Q water for 5 min and then activated electrochemically by cycling in 0.1 M H_2SO_4 (−0.20 to 1.6 V, scan rate 100 mV/s, 50 cycles) using a computer-controlled FAS2 potentiostat (Gamry Instruments, Warminster, PA, USA). It was then washed thoroughly with Milli-Q water.

The polished, activated electrode was introduced into the flow-through wall-jet cell (4) in a Teflon holder and adjusted to a distance of about 2 mm from the inlet nozzle. The carrier solution (PBS) was pumped through the system at a flow rate of 500 μL/min. The electrochemical cell was connected to a single line of the flow injection system, in which the carrier flow was maintained with a peristaltic pump (Minipuls 2, Gilson, Villiers, France). The samples were loaded and injected using a LabPRO six-port injection valve (Rheodyne, Cotati, CA, USA) equipped with a 50-μL injection loop. A three-electrode configuration was used for amperometric detection: the Pt working electrode, a Ag/AgCl reference electrode (Beta Sensor AB, Lund, Sweden) in a chamber containing 0.1 M KCl, and a Pt wire counter electrode. All electrodes were connected to one ZÄTA LC4B potentiostat (Zäta Elektronik, Höör, Sweden), and a potential of 0.4 V was applied. The current peaks obtained were recorded using a pen recorder (Kipp & Zonen, Delft, The Netherlands).

Electrochemical measurements

FIA was initiated by flushing PBS through the analytical system. After approximately 10 min when a stable baseline was observed, the sample was added to the continuous flow of PBS. The peak response was recorded within approximately 30 s, which is the time required for the whole sample (50-μL injection loop) to reach/pass the electrode surface at a flow rate of 500 μL/min. Each sample was analyzed in triplicate with a sample throughput of 2 samples/min. Six individual current responses were obtained from two independent batch cultures of *C. saccharolyticus* ($N = 2$, $n = 3$). The experimentally acquired response (mA) was converted to FC reducing capacity (FRC; mM) (Heiskanen et al. 2009).

To avoid fouling of the electrode, the working electrode was cleaned and re-activated regularly after 90 measurements using the previously described procedure (Heiskanen et al. 2009).

Effect of oxamate on intracellular redox state

Exponentially growing cells were harvested as described above and re-suspended in anaerobic PBS to a final cell mass of 5 g/L. The incubation and electrochemical assays were carried out as described above, but with the addition of the inhibitor (10 mM sodium oxamate). Experiments were performed in biological duplicate.

Detection of hydrogen productivity by *C. saccharolyticus*

The gas in the headspace of the bioreactor was analyzed to determine the amounts of CO_2 and H_2 using a Varian CP-4900 Micro Gas Chromatograph (Varian, Middelburg, The Netherlands) equipped with a thermal conductivity detector (100 mA). The results were analyzed using Galaxie Chromatography Software (v.1.9.3.2). The productivity was calculated as previously described (de Vrije et al. 2007).

Statistical analysis of the data

Statistical comparisons were performed using two-way ANOVA (GraphPad Software, San Diego, CA, USA). The reproducibility of the assay results is presented as the average and the standard error of the mean (SEM) calculated by GraphPad Prism software.

Bioinformatics analysis

Candidates for membrane-associated oxidoreductases were defined based on sequence similarity. The criteria for the search were that the candidate should have an NADH-binding domain and possess either a signal peptide or a transmembrane region. The genome of *C. saccharolyticus* was analyzed with regard to NADH-binding domain signatures using a Hidden Markow Model available in the IntePro database (http://www.ebi.ac.uk/interpro/). Signal peptide and transmembrane region analysis of the selected genes was performed using the signal peptide and transmembrane region viewers available at the IMG database (http://img.jgi.doe.gov).

Results and discussion

Applicability of the method for probing the redox metabolism in *C. saccharolyticus*

The applicability of the assay at optimal conditions for *C. saccharolyticus* (strict anaerobic conditions and 70°C) was assessed. Anaerobiosis was maintained by using sealed serum flasks and handling the cells in an anaerobic chamber. Primarily, the applicability of the mediator system at 70°C was evaluated. Two different sets of experiments were performed, i.e., a combined mediator system with menadione and FC, and a single mediator system with

FC only. FC was selected due to its reported thermo-stability (Chaubey and Malhotra 2002; Harwood and Pouton 1996) and menadione for its ability to enter the cell (Kostesha et al. 2009a; Spegel et al. 2007).

Figure 3 shows the stability of the mediator system in the assay condition of *C. saccharolyticus*. The signal was equal or higher when FC was used as a sole mediator, indicating that menadione did not contribute to the signal. The signal of the system with FC as sole mediator increased for 60 min. The increased signal is due to formation of the reduced cofactor as a result of sugar oxidation. The observation that the signal did not decrease indicates that the system was stable to exposure to 70°C for 60 min in the assay mixture (Fig. 3). In addition, after 20 min, the electrochemical signal was significantly higher in the FC alone system than in the combined system. This result demonstrates that menadione has a negative effect on the system and is, therefore, not an appropriate mediator in this condition with *C. saccharolyticus*. A similar negative effect of menadione was observed by Kim et al. (2006). Based on this observation (Fig. 3) and the fact that FC did not react spontaneously with hydrogen (data not shown), FC as sole mediator was selected for the continuation of the experiments.

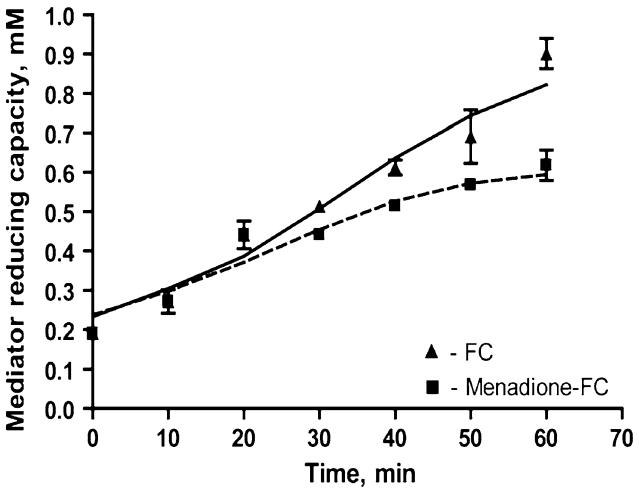

Fig. 3 Concentration–time response curves of FC and menadione-FC oxidation on a Pt electrode at 70°C under anaerobic conditions. The figure shows the FC and menadione reduction capacity by intact cells of *C. saccharolyticus* when glucose was added

However, since the FC/FoC couple is hydrophilic and does not penetrate the cell membrane (Chaubey and Malhotra 2002; Harwood and Pouton 1996), FC needs to be reduced on the membrane surface by membrane-associated enzymes for any reaction to take place. We therefore conducted a bioinformatics survey of redox-dependent enzymes (oxidoreductases) in *C. saccharolyticus* that contain a signal peptide or a trans-membrane region. Indeed, *C. saccharolyticus* possesses several oxidoreductases that contain either property, such as the previously annotated ferredoxin-dependent Ni–Fe hydrogenase (van de Werken et al. 2008) and various NAD(H)-dependent dehydrogenases (Table 1). Based on sequence similarity, it is therefore the turnover of these two redox carriers (Fd_{red} and NADH) that are measured in the assay.

Sugar metabolism and corresponding difference in the electron flow

To determine the effect of various sugars on the intracellular electron flow, *C saccharolyticus* was harvested in the exponential growth phase and supplemented with glucose, sucrose, or xylose (Fig. 4). An increase in FoC concentration was observed after 10 min of incubation with each sugar, and the rate decreased slightly after 50 min in all cases (Fig. 4). This indicates that NADH and/or Fdr_{ed} formation through xylose, glucose, or sucrose consumption provoked a redox signal that was easily detectable using amperometry. The addition of xylose led to a considerably stronger signal than sucrose and glucose, and the difference between glucose and sucrose metabolism was significant at the 95% confidence level, according to a two-way ANOVA test. The observation regarding xylose and glucose indicates that the flux through the pathway was higher on xylose than on glucose. This observation correlates well with a higher growth rate on xylose than on glucose (Table 2). The growth rate on xylose was 1½ times faster than on sucrose and glucose, which is consistent with the

Table 1 Identification of membrane-associated dehydrogenases in *C. saccharolyticus*

Predicted function	Locus tag	Membrane marker	Binding domain	Reference
Ni–Fe hydrogenase	Csac_1534-1539	Transmembrane helix	Fd	van de Werken et al. (2008)
2-Deoxy-D-gluconate 3-dehydrogenase	Csac_2718	Signal peptide	NAD(H)	This study
Alcohol dehydrogenase GroES domain protein	Csac_1226	Transmembrane helix	NAD(H)	This study
Fumarate reductase/succinate dehydrogenase flavoprotein domain protein	Csac_1640	Transmembrane helix, signal peptide	NAD(H)	This study
Lactate dehydrogenase	Csac_1027	Transmembrane helix, signal peptide	NADH	This study

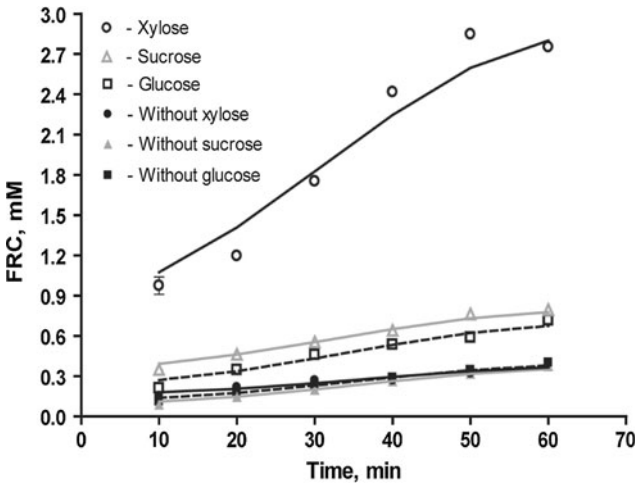

Fig. 4 Concentration–time response curves of ferrocyanide oxidation on a Pt electrode at an applied potential of +400 mV. The figure shows the ferricyanide reduction rate of intact cells of *C. saccharolyticus* harvested in the exponential growth phase. *Filled symbols* denote the absence of sugar, whereas *open symbols* denote the presence of sugar (10 g/L). Xylose (*open circles*), sucrose (*open triangles*), or glucose (*open squares*). The results are presented as the average and SEM of two biological and three technical replicates. The errors are too small to be seen on this scale

Table 2 Growth rate and FC reduction rate divided by the growth rate from controlled batch cultures of *C. saccharolyticus* on various sugars (10 g/L)

	Growth rate (h^{-1})	$\Delta FC/\mu$ (mM)	Reference
Sucrose	0.23 ± 0.02	3	Willquist et al. (2009)
Glucose	0.24 ± 0.01	3	Willquist et al. (2009)
Xylose	0.35 ± 0.02	8	This study

± indicates the 95% confidence interval

amperometric results. However, the effect of carbon source was more apparent with the amperometric assay than when just considering the growth rate (Table 2). In addition, the sugar uptake rate on xylose is reported to be higher than on glucose (van Fossen et al. 2008). *C. saccharolyticus* can co-metabolize different sugars using ABC transporters, but the substrate consumption rate differs due to the variation in affinity for each sugar and because more transporters are upregulated on xylose than on glucose, i.e., xylose is preferred over glucose, resulting in a faster uptake rate (van Fossen et al. 2008). Consequently, a faster electron flow was detected on xylose than on glucose.

To investigate whether this assay also measures the cellular redox state, control experiments were performed without the addition of an external carbon source. This experiment gave the same FRC signal regardless of the

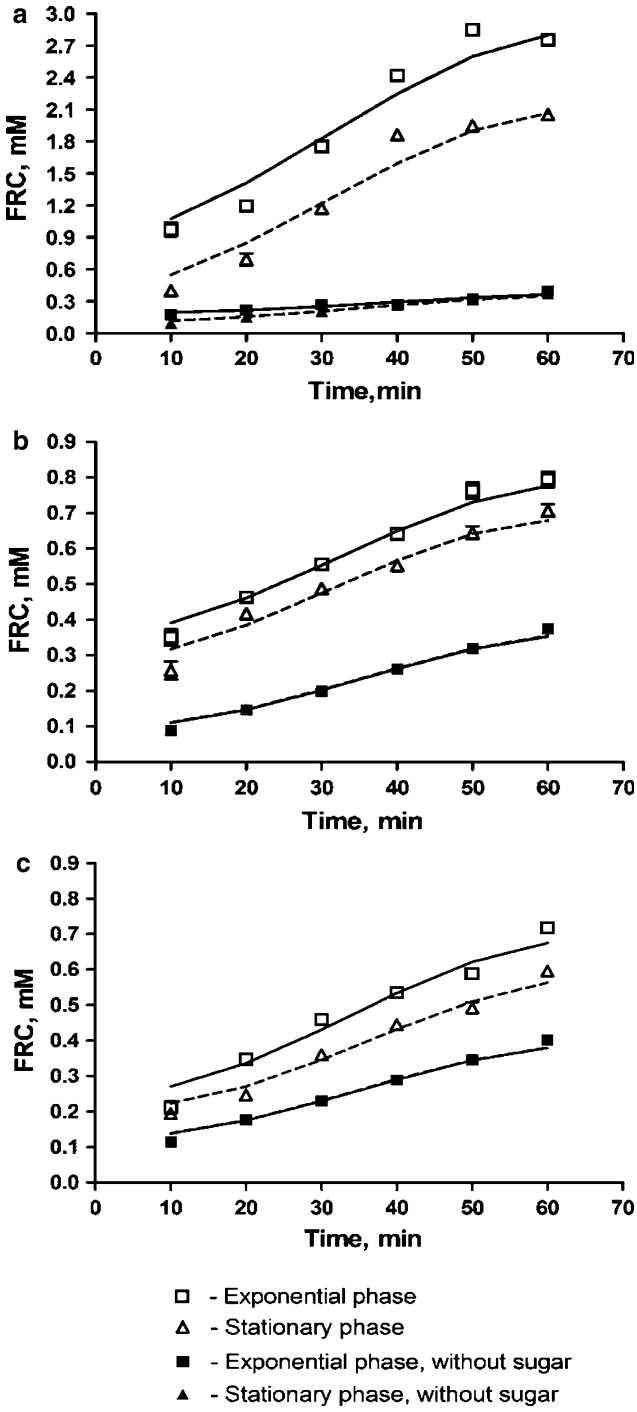

Fig. 5 Concentration–time response curves of ferrocyanide oxidation on a Pt electrode. The figure shows the ferricyanide reduction rate by enzymes within the intact cells of *C. saccharolyticus* when xylose (**a**), sucrose (**b**), or glucose (**c**) was added. Cells were harvested in the exponential (*open squares*) and stationary (*open triangles*) phases. *Filled symbols* denote the absence of sugar, whereas *open symbols* denote the presence of sugar (10 g/L). Results are presented as the average and SEM of two biological and three technical replicates. The errors are too small to be seen on this scale

sugar used in the medium from which the exponentially growing cells were extracted (Fig. 4). This indicates that the assay measures the electron flow caused by substrate oxidation and not the cellular redox status in the harvest cells; hence, the assay is not sensitive enough to measure the redox status in these cells.

Lactate formation in the transition to the stationary phase

We have recently reported that metabolism is party directed toward lactate formation in the transition to the stationary phase, coinciding with a decrease in the glucose and acetate fluxes (Willquist and van Niel 2010). The mediated amperometric method developed in the present study was applied to investigate the competitive behavior between pyruvate ferredoxin oxidoreductase (PFOR) and lactate dehydrogenase (LDH) for pyruvate (Fig. 2), and the difference in electron flow in growing cells in the exponential and stationary phases. The observed electrochemical response from cells harvested in the early stationary growth phase was significantly lower than that from cells harvested in the exponential growth phase (Fig. 5; Table 3). This finding indicates that the NADH- and Fd_{red}-surplus flux (generation–consumption) diminishes in the stationary phase compared to exponentially growing cells. Hence, the lower FRC in the stationary phase is due to a lower substrate uptake rate or glycolytic flux, or that less pyruvate is directed to acetate in cells from the stationary growth phase, or a combination of both. Indeed, our recent results have shown that both the substrate consumption rate and the acetate formation rate are higher in exponentially growing cells, while lactate formation is induced in the stationary phase (Willquist and van Niel 2010). This coincided with a twofold increase in the LDH activity, while the PFOR activity remained the same in the two growth phases (Willquist and van Niel 2010). Cells that were harvested from the exponential phase were thus adapted to a higher substrate uptake and oxidation rate and exhibited a lower LDH activity (Willquist and van Niel 2010), generating a higher amperometric signal. In contrast, cells from the stationary phase had a lower substrate uptake and oxidation rate, and a higher ratio of LDH/PFOR activity ratio (Willquist and van Niel 2010), resulting in an overall lower amperometric signal.

Without the addition of sugar, the electrochemical responses of cells harvested in the exponential and early stationary growth phases were not significantly different at the 95% confidence interval (Fig. 5), according to the two-way ANOVA test. However, we have previously shown that the intracellular redox state is affected by the growth phase (Willquist and van Niel 2010). Again, this result confirms that the developed method is complementary to the spectrophotometer method. Herein, the internal turnover of NADH and Fd_{red} is measured, while the conventional spectrophotometric method determines the state of separate redox carriers.

Oxidation of NADH by cytosolic and membrane-associated enzymes

The results presented above show that the electron flow is different in cells in the exponential or stationary phase. To evaluate the impact of NADH-dependent dehydrogenases on the electron flow, sodium oxamate was used as an inhibitor. Sodium oxamate is known to inhibit NADH-dependent LDH, because it is an analog of pyruvate (Bloomfield and Alberty 1963). By employing mediated amperometry, we were able to detect a clear difference in the electron flow when oxamate (100 μM) was supplied to cells with and without the addition of various carbon sources (Fig. 6), since more NADH was available for FC reduction. The electrochemical signal was at least 3 times higher when oxamate was added to cells than in its absence (Table 3).

This demonstrates that oxamate has a strong inhibitory effect on the NADH-dependent LDH in *C. saccharolyticus*.

Table 3 FC reducing capacity (FRC) (mM) of intact *C. saccharolyticus* cells (0.5 g/L) obtained after 10 and 60 min of incubation, with and without oxamate, in the presence of different carbon sources

Conditions		Growth phase			
		Exponential phase		Stationary phase	
Carbon source	Incubation time	10 min	60 min	10 min	60 min
Xylose	No oxamate	0.97 ± 0.10	2.83 ± 0.02	0.40 ± 0.01	2.05 ± 0.02
	100 μM oxamate	0.86 ± 0.10	9.28 ± 0.01	ND	ND
Sucrose	No oxamate	0.35 ± 0.02	0.79 ± 0.03	0.26 ± 0.03	0.65 ± 0.02
	100 μM oxamate	0.87 ± 0.03	3.48 ± 0.02	ND	ND
Glucose	No oxamate	0.21 ± 0.02	0.70 ± 0.01	0.20 ± 0.02	0.54 ± 0.02
	100 μM oxamate	0.63 ± 0.02	2.97 ± 0.02	ND	ND

ND not determined

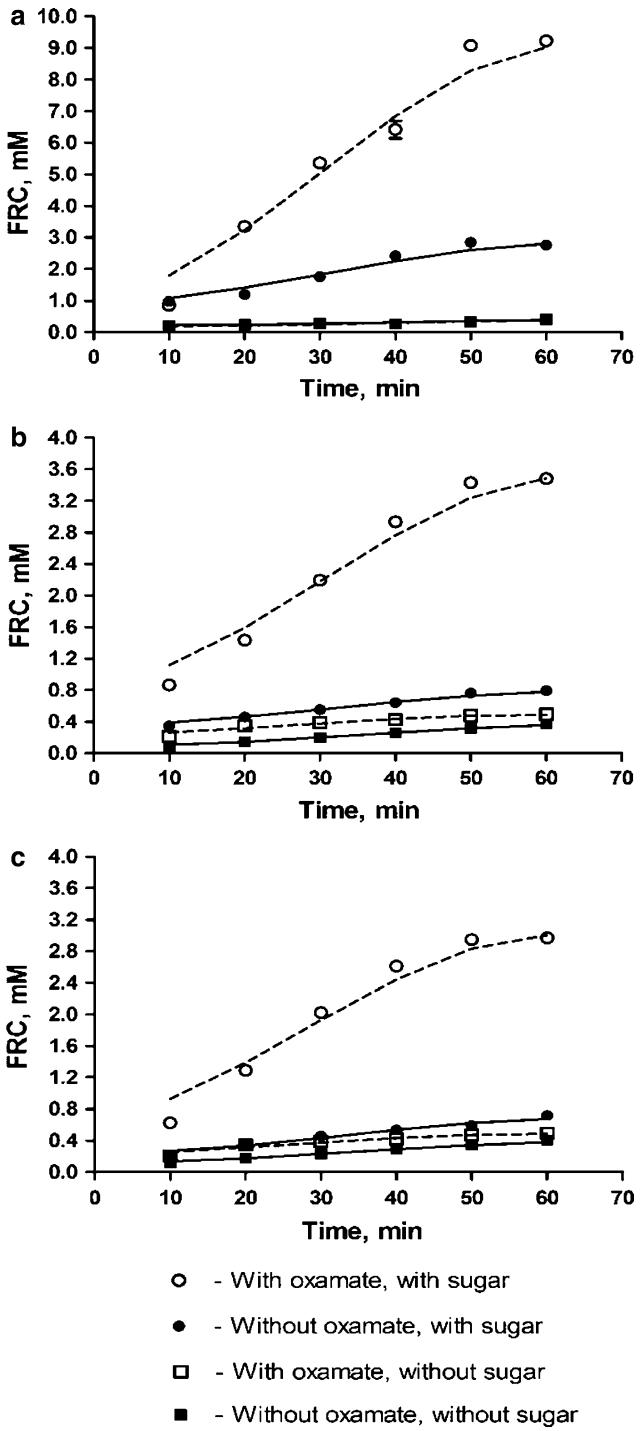

Fig. 6 Concentration–time response curves of FoC oxidation in the presence of oxamate at a concentration of 100 μM (*open symbols*), and responses obtained without oxamate (*filled symbols*), when xylose (**a**), sucrose (**b**), or glucose (**c**) was added to *C. saccharolyticus*. Results are presented as the average and SEM of two biological and three technical replicates. The errors are too small to be seen on this scale

It also illustrates the strong competition between these cytosolic dehydrogenases for the electrons available for FC reduction by membrane proteins. In addition, the considerable impact of NADH-dependent dehydrogenases on the electron flow in *C. saccharolyticus* is demonstrated.

Conclusions

In this paper, we describe a noninvasive, FC-mediated amperometric method able to accurately detect the difference in the electron flow under strictly anaerobic and extremely thermophilic conditions. The method offers several advantages over traditional methods of probing the redox profile, i.e., (1) it rapidly provides important information about the intracellular metabolism in strictly anaerobic, extremely thermophilic cells; (2) it is noninvasive and can therefore be performed on intact cells; (3) strict anaerobic conditions can be maintained during the assay; (4) a high temperature (70°C) was achieved during the incubation period; and (5) FC was shown to be a stable redox mediator, which could not penetrate the cell membrane, but was able to rapidly exchange electrons from redox enzymes located in the plasma membrane.

The versatility of the method was demonstrated by studying the response of *C. saccharolyticus* cells supplied with various carbon sources: xylose, sucrose, or glucose. In addition, we were able to reveal significant differences in the electron flow in cells harvested during the exponential and the early stationary growth phases, and to probe the impact of NADH-dependent dehydrogenases, such as LDH. It was possible to elucidate differences in sugar metabolism and growth phase in this bacterium without disrupting the cells.

The outcome of the noninvasive experiment was that the flux through the EMP is greatly dependent on the sugar used, which is coherent with the observation of a higher growth rate on xylose and the previously observed higher substrate uptake rate on xylose than on glucose (van Fossen et al. 2008). Moreover, the result demonstrates that although the carbon sources are metabolized in the same pathways, the sugar uptake rate, the flux distribution at the pyruvate node, and the regulation of these pathways differ depending on the sugar.

In addition, by using this amperometric method, we were able to demonstrate that the reduced cofactor-generating flux was lower in cells from stationary growth than in cells from exponential growth. This is consistent with our previous observation of a higher substrate uptake rate, higher glycolytic flux, and lower LDH activity in exponentially growing cells (Willquist and van Niel 2010).

Finally, we show that sodium oxamate is a strong inhibitor of NADH-dependent LDH in *C. saccharolyticus* directing the electron flux to membrane-associated enzymes. By using sodium oxamate, the competitive behavior of the cytosolic and membrane-associated oxidoreductases for the

redox carriers was illustrated. The method can therefore be applied in future studies to monitor the regulation of these NADH-dependent enzymes under different physiological conditions by using different specific inhibitors.

Overall, the results presented here demonstrate that this amperometric method is a powerful tool for probing the metabolism in *C. saccharolyticus*, enabling the detection of cytosolic dehydrogenase activity and elucidating its consequence on electron flow in *C. saccharolyticus*.

Furthermore, the methodology could be applied to probe the electron flow in other extremely thermophilic, strictly anaerobic cells to increase our fundamental knowledge of these intriguing microorganisms.

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