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Unbalanced fermentation of glycerol in *Escherichia coli* via heterologous production of an electron transport chain and electrode interaction in microbial electrochemical cells

Katrin Sturm-Richter [a], Frederik Golitsch [a], Gunnar Sturm [a], Elena Kipf [b], André Dittrich [c], Sebastian Beblawy [a], Sven Kerzenmacher [b], Johannes Gescher [a,*]

[a] Institute for Applied Biosciences, Department of Applied Biology, Karlsruhe Institute of Technology, Fritz-Haber-Weg 2, 76131 Karlsruhe, Germany
[b] Laboratory for MEMS Applications, IMTEK – Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, D-79110 Freiburg, Germany
[c] Institute of Photogrammetry and Remote Sensing, Englerstraße 7, D-76131 Karlsruhe, Germany

HIGHLIGHTS

- Anaerobic glycerol fermentation in *Escherichia coli* can be accelerated to an anode.
- Functional screen for heterologous cytochromes with respiratory activity in *E. coli.*
- Development of an inducible methylene blue-assisted anode respiration system.
- New electron transport chain contains the cytochromes CymA, MtrA and STC.

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ABSTRACT

Microbial electrochemical cells are an emerging technology for achieving unbalanced fermentations. However, organisms that can serve as potential biocatalysts for this application are limited by their narrow substrate spectrum. This study describes the reprogramming of *Escherichia coli* for the efficient use of anodes as electron acceptors. Electron transfer into the periplasm was accelerated by 183% via heterologous expression of the *c*-type cytochromes CymA, MtrA and STC from *Shewanella oneidensis.* STC was identified as a target for heterologous expression via a two-stage screening approach. First, mass spectroscopic analysis revealed natively expressed cytochromes in *S. oneidensis*. Thereafter, the corresponding genes were cloned and expressed in *E. coli* to quantify periplasmic electron transfer activity using methylene blue. This redox dye was further used to expand electron transfer to carbon electrode surfaces. The results demonstrate that *E. coli* can be reprogrammed from glycerol fermentation to respiration upon production of the new electron transport chain.

1. Introduction

In microbial fuel cells (MFCs) microbes use anodes as electron acceptors. In contrast to all other respiratory electron sinks that can be utilized by microorganisms, anodes cannot be depleted. Thus, they offer the biotechnological possibility of developing anaerobic pathways that do not need to be stoichiometrically balanced with respect to the oxidation states of substrates and products (Flynn et al., 2010). By this, unbalanced fermentation represents a new biotechnological tool for broadening the spectrum of efficiently producible platform chemicals. Butanediol, itaconic acid, and terpenes are examples of industrially important compounds that are more oxidized than prevalent substrates like glucose or glycerol and therefore cannot be produced as the only end product under anoxic fermentative conditions. Aerobic production routines could be an option for the production of these compounds, but are often accompanied by losses due to high anabolic substrate consumption. Hence, the benefit of the increased catabolism that prevails under anoxic conditions can be exploited by using electrodes as electron acceptors. Since microorganisms catalyze the electron transfer on the anodes by aid of enzymes it is possible to use carbon-based electrode materials like graphite felt. Biotechnological important features of such anodes materials are for example their relatively low price compared to noble element electrodes that have to be used in conventional hydrogen fuel

* Corresponding author. Tel.: +49 721 608 41940; fax: +49 721 608 41941.
E-mail address: johannes.gescher@kit.edu (J. Gescher).

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cells. Moreover, the use of anodes as electron acceptors is connected to the beneficial production of electric energy, which might in the future lead to an energy autarkic control of process parameters.

Anode respiration is dependent on extended respiratory chains that connect intracellular oxidation processes to cell surface-localized terminal electron transfer reactions. Certainly, evolution did not select for anode-reducing organisms, but the electrode mimics the surface of insoluble electron acceptors, such as ferric and manganese oxides (Richter et al., 2012).

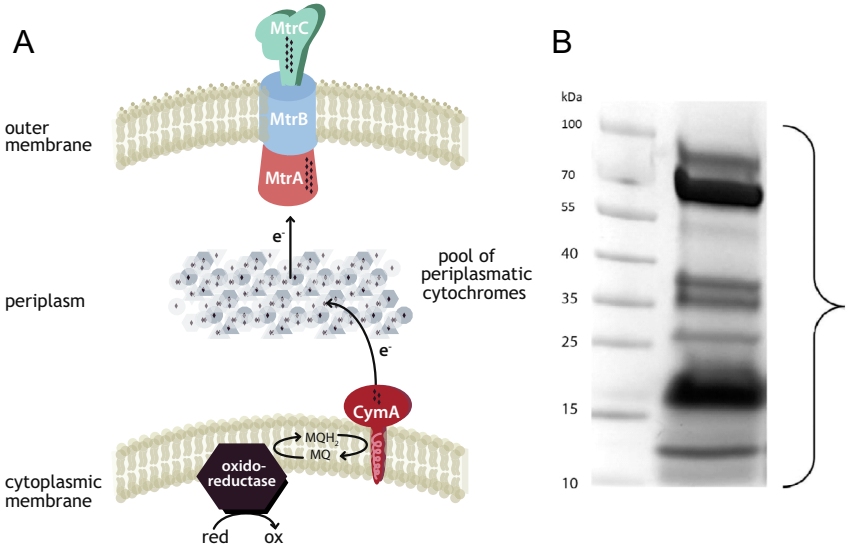
The number of isolated species that can conduct extracellular respiration is steadily rising (Sturm et al., 2012). Nevertheless, the proteobacteria *Shewanella oneidensis* and *Geobacter sulfurreducens* remain the model organisms for studying (i) the biochemistry that enables extracellular respiration and (ii) the physiology of microbes with extended respiratory chains. For both organisms, multiple lines of evidence suggest that *c*-type cytochromes play a major role as electron transfer proteins (Shi et al., 2007). Interestingly, the minimal set of proteins required for transporting electrons to the cell surface has not yet been determined. This lack of knowledge is likely due to (i) the enigmatic co-expression of multiple *c*-type cytochromes under anoxic conditions, (ii) the apparent interconnection of multiple electron transfer pathways and (iii) the redundant activity of some of the expressed cytochromes. However, some proteins are indispensable for ferric iron and anode reduction in *S. oneidensis* (Fig. 1A; Bretschger et al., 2007; Gao et al., 2010).

The tetraheme *c*-type cytochrome CymA directs electrons from the menaquinol pool to several reductases in the periplasm. Hence, *cymA* mutants are unable to use a multitude of anoxic electron acceptors, including ferric iron and anodes (Myers and Myers, 1997; Schwalb et al., 2003). Beyond CymA, MtrA is the only other periplasmic cytochrome that is required for iron and anode respiration (Bretschger et al., 2007; Schicklberger et al., 2013). Furthermore, among the array of expressed periplasmic *c*-type cytochromes, two proteins are produced in larger amounts (Fonseca et al., 2013). Surprisingly, one of these proteins is the respiratory fumarate reductase of *S. oneidensis* (FccA), which in contrast to the fumarate reductases of other organisms, is a soluble periplasmic heme-flavoprotein. The small tetraheme cytochrome STC (SO2727) is the second highly expressed periplasmic *c*-type cytochrome. The exact role of this protein in iron or anode reduction remains uncertain, although detailed studies regarding the structure and electrochemical properties of this protein have been published (Fonseca et al., 2009; Paquete et al., 2010; Qian et al., 2011).

The restricted substrate spectrum of *S. oneidensis* is a major limitation in the context of MFC applications. This organism uses only typical fermentation end products as electron donors under anoxic conditions (Scott and Nealson, 1994). This behavior excludes the utilization of cheap and industrially important carbon sources, such as pentose and hexose sugars. In fact, a restricted carbon spectrum is a typical characteristic of most exoelectrogenic species. This disadvantage could be obviated by connecting a metabolically versatile and genetically tractable bacterium, such as *Escherichia coli,* to anodes.

Besides pentose and hexose sugars, glycerol is an interesting substrate for biotechnological production processes. It is a byproduct of biodiesel production and massive growth of the biodiesel industry has led to a large surplus of this compound and to very low crude glycerol prices. However, fermentation of glycerol is not trivial. Due to its highly reduced state, traditional fermentation routines lead to product mixtures, when more oxidized chemicals are supposed to be the desired end-product. Also, fermentation of glycerol was thought to be restricted to only a few species. Recently it was discovered that *E. coli* can grow fermentatively on this substrate (Dharmadi et al., 2006). Still, under fermentative conditions *E. coli* produces mainly ethanol from glycerol and higher oxidized products can only be obtained by the addition of an external electron acceptor. An anode as electron sink can overcome these limitations and electrofermentation is a promising new tool to expand the product spectrum of anoxic glycerol fermentation.

This study describes a new and accelerated way for anaerobic glycerol fermentation by connecting the central metabolism of



c-type cytochrome	kDa	score
SO1421 IfcA	60.6	533
SO0970 FccA	59.8	755
SO3980 NrfA	49.7	21
SO4047 SoxA	36.2	102
SO2178 CcpA	34.0	383
SO1777 MtrA	32.4	38
SO4666	19.9	17
SO4048	19.3	21
SO4485	16.5	30
SO3420	14.3	148
SO2727 STC	9.7	137
SO0264 ScyA	8.9	26

Fig. 1. Periplasmic *c*-type cytochromes expressed under iron reducing conditions in *S. oneidensis* MR-1. (A) General overview of components involved in the dissimilatory reduction of iron in *S. oneidensis*. The menaquinol oxidase CymA transports respiratory electrons into the periplasm. Here, a pool of different *c*-type cytochromes exists that can be reduced by CymA. So far it is unknown whether a specific electron transport pathway through the periplasm exists or it is a rather stochastic process in which a multitude of *c*-type cytochromes are involved. Electrons are transported through the outer membrane via a three protein complex, consisting out of the two cytochromes MtrA and MtrC and the connecting β-barrel protein MtrB. MtrC is the terminal reductase of the electron transport chain to ferric iron or an anode. (B) Heme stain of a periplasmic protein fraction derived from *S. oneidensis* cells grown under ferric iron reducing conditions. The periplasmic fraction was either used for MudPIT or MS analysis. Heme-staining positive bands were furthermore excised from the gel and directly analyzed by mass spectrometry. All identified cytochromes and their corresponding sizes and scores are listed in the table. Values of protein masses are deduced from primary sequence.