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Heterologous expression and purification of a multiheme cytochrome from a Gram-positive bacterium capable of performing extracellular respiration

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ABSTRACT

Microbial electrochemical technologies are emerging as environmentally friendly biotechnological processes. Recently, a thermophilic Gram-positive bacterium capable of electricity production in a microbial fuel cell was isolated. *Thermincola potens* JR contains several multiheme *c*-type cytochromes that were implicated in the process of electricity production. In order to understand the molecular basis by which Gram-positive bacteria perform extracellular electron transfer, the relevant proteins need to be characterized in detail. Towards this end, a chimeric gene containing the signal peptide from *Shewanella oneidensis* MR-1 small tetraheme cytochrome *c* (STC) and the gene sequence of the target protein TherJR_0333 was constructed. This manuscript reports the successful expression of this chimeric gene in the Gram-negative bacterium *Escherichia coli* and its subsequent purification and characterization. This methodology opens the possibility to study other multiheme cytochromes from Gram-positive bacteria, allowing the extracellular electron transfer mechanisms of this class of organisms to be unraveled.

Introduction

Microorganisms capable of performing extracellular electron transfer hold great potential for the development of environmentally friendly biotechnological applications. These are generally known as microbial electrochemical technologies of which microbial fuel cells (MFCs)[1] are the best known example. These technologies have the potential to significantly improve the production of sustainable and renewable energy, wastewater treatment processes, and implementation of sustainable biorefinery processes [1,2]. Both Gram-negative and Gram-positive bacteria are known to successfully transfer electrons directly to a solid anode in an operating MFC [3]. Studies performed on these devices showed that thermophiles produce higher levels of current than mesophiles in the same reactor, being often the prevalent electricity generating communities in the anode [4–6]. Recently a thermophilic Gram-positive bacterium, *Thermincola potens* JR, was isolated in an operating MFC [7].

Studies exploring electron transfer in MFCs have taken place mainly on mesophilic Gram-negative bacteria, where the best understood electron transfer pathway is from the gamma proteobacterium *Shewanella oneidensis* MR-1. In this organism multiheme *c*-type cytochromes (MHCs) transfer electrons from cytoplasmic and inner-membrane oxidizing enzymes towards redox super-complexes at the cell surface that are responsible for the reduction of solid phase electron acceptors [8]. When compared with Gram-negative bacteria, Gram-positive bacteria lack the outer membrane and present a thicker cell wall made of peptidoglycan. Moreover, the width of the periplasmic space between the membrane and the cell wall is smaller than the typical periplasm of Gram-negative bacteria [5,9,10]. These differences suggest a different electron transfer mechanism towards the cell surface. Interestingly, like the well-studied Gram-negative bacteria *S. oneidensis* MR-1 and *Geobacter sulfurreducens* [11], the genome of *T. potens* JR contains 32 genes that code for putative *c*-type cytochromes [12]. The physiological characterization of *T. potens* JR revealed that it is able to reduce insoluble ferric compounds [7]. Furthermore, a recent study by Carlson and co-workers showed biochemical and biophysical evidence that MHCs are implicated in the reduction of insoluble ferric compounds [13]. However, to

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[1] *Abbreviations used:* STC, small tetraheme cytochrome *c*; MFCs, multiheme *c*-type cytochromes; N-region, N-terminal region; H-region, hydrophobic region; TB, Terrific Broth; DEAE, diethylaminoethyl; DDM, n-Dodecyl-β-maltopyranoside.

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explore the mechanisms by which Gram-positive bacteria perform extracellular electron transfer it is necessary to characterize in detail the proteins involved in this process.

Escherichia coli is by far the best studied heterologous expression system for recombinant proteins [14,15]. However the heterologous expression of *c*-type cytochromes requires additional care because it relies on specific cellular machinery to ensure the covalent binding of the heme to the apo-cytochrome. This process depends on the cytochrome *c* biogenesis system that ensures the translocation of the heme to the periplasmic space and the covalent heme attachment to the apo-cytochrome [16,17]. The native cytochrome *c* maturation system, coded in *E. coli* by the gene cluster *ccm*ABCDEFGH [16–18] does not operate under aerobic conditions [19]. Therefore, co-expression of this system with the target *c*-type cytochrome is necessary for the proper maturation of the recombinant *c*-type cytochrome under aerobic conditions [20–24].

In addition to the cytochrome *c* maturation system, the successful production of recombinant *c*-type cytochromes also depends on the translocation of the apo-protein to the periplasmic space where the heme assembly occurs [17,18]. The translocation to the periplasmic space is possible due to the presence of a specific signal peptide sequence. This signal sequence generally contains 15–30 residues and consists of three specific regions: the positively charged N-terminal region (N-region), the hydrophobic region (H-region) and the neutral C-region at the C-terminal end that is recognized by the signal peptidases in the periplasmic space [25,26]. Although for both Gram-positive and Gram-negative bacteria the machinery of the Sec pathway responsible for the recognition of the hydrophobic N-terminal leader sequence and translocation of the apo-protein are very similar, the cleavage of this sequence by peptidases is quite distinct [25,27]. While in Gram-negative bacteria the signal peptidase cleavage occurs three to seven residues from the C-terminal end of H-region, in Gram-positive bacteria this cleavage takes place preferentially from seven to nine residues from the same position. Therefore, special care has to be taken in the heterologous expression of Gram-positive *c*-type cytochromes in Gram-negative bacteria like *E. coli*.

In this paper, we present, for the first time, the heterologous expression and purification of a multiheme *c*-type cytochrome from a Gram-positive bacterium. In order to ensure the proper cleavage of the target protein, the signal peptide of the periplasmic decaheme protein ThrJR_0333 from *T. potens* JR was replaced with the signal peptide from the small tetraheme cytochrome *c* from *S. oneidensis* MR-1 (SO_2727), an abundant constitutively expressed protein [28–30]. This approach enabled the over-expression of the Gram-positive decaheme cytochrome TherJR_0333 in the Gram-negative bacterium *E. coli*. This methodology will facilitate studies aimed at elucidating the mechanisms of extracellular electron transfer performed by Gram-positive bacteria that colonize anodes, and will contribute to the understanding and improvement of microbial electrochemical technologies such as MFCs.

Materials and methods

Bacterial strains and growth conditions

The sequence of the signal peptide derived from the small tetraheme cytochrome *c* (STC) from *S. oneidensis* MR-1 was used to produce a chimeric gene with *therJR_0333* gene from *T. potens* [29], using the primers listed in Table 1 (NZYtech, Portugal). The *stcsp*_FW primer was designed according to Shi et al. [31] to clone efficiently the chimeric gene (named *stcsp_therJR0333*) into pBAD202/D-TOPO vector (Invitrogen, USA). Cloning was performed according to manufacturer specifications, and the final expression vector (pCP01) was inserted into the *E. coli* strain JM109 (DE3) co-transformed with vector pEC86, which contains the *ccm*ABCDEFGH genes [32]. Positive transformants were used for expression tests, where different media, induction times and inducer concentrations were tested. Bug-buster reagent (Novagen, USA) was used to check the best over-expression condition. The selected condition was later used to over-express TherJR_0333. Briefly, the transformants were grown in Terrific Broth (TB) with 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol in 5 L Erlenmeyer flasks containing 2 L of medium and 1% of inoculum at 37 °C and 150 rpm. At mid-log phase (about 6 h of growth) protein expression was induced with 1 mM of arabinose, the temperature was lowered to 30 °C and cells were allowed to grow for additional 16 h. Cells were harvested by centrifugation at 10 000*g* for 10 min at 4 °C.

Protein purification

The cell pellet was suspended in an osmotic shock solution (0.5 M sucrose, 0.2 M Tris–HCl, 0.5 mM EDTA and 100 mg L^{-1} lysozyme, pH 7.6) containing protease inhibitor cocktail (Sigma) and DNase I (Sigma) [33]. This mixture was incubated at 4 °C for 30 min with gentle stirring. The supernatant containing the periplasmic fraction was cleared by ultracentrifugation at 20 000*g* for 1 h at 4 °C, dialyzed overnight against 20 mM Tris–HCl (pH7.6) and concentrated in an ultrafiltration cell with a 10 kDa cut-off membrane. The resulting sample was loaded onto an ion exchange diethylaminoethyl (DEAE) column (GE Healthcare) previously equilibrated with 10 mM Tris–HCl (pH 7.6). The resulting fractions were eluted with a salt gradient from 0 to 1 M KCl in the same buffer. The chromatographic fractions were followed by UV–visible spectroscopy and SDS–page (12%) to select those containing the protein of interest. The target protein, TherJR_0333, was eluted at 10 mM Tris–HCl. The fractions containing TherJR_0333 were concentrated and analyzed by UV–visible spectroscopy and SDS–PAGE (12% gel) to check for the presence of further contaminating proteins. Over time, the fraction containing TherJR_0333 protein started to form a red precipitate. Solubilization of this precipitate was achieved using 10 mM potassium phosphate buffer (pH7.6) with 100 mM potassium chloride and 0.05% of the non-ionic detergent n-Dodecyl-β-maltopyranoside (DDM). Since the addition of the detergent prevent further precipitation of the target protein, the later characterization was performed with solubilized protein using 0.05% DDM. A single band on the SDS–Page gel confirmed the purity of the target protein, and the purity index of the sample was defined by $A_{Soret\ peak}/A_{280nm}$ ratio.

TherJR_0333 identification by mass spectrometry

The data were acquired in positive linear MS mode using a 4800plus MALDI-TOF/TOF (AB Sciex) mass spectrometer and using

Table 1
Oligonucleotides used to construct the chimeric gene.

stcsp_FW	CACCTAAGAAGGAGATATACATCCCGTGAGCAAAAAACTATTAAG
therJR0333_RV	GTGTTAAAAAGGCTACATAAATTTTCCTTGAAAAAGGTTATG
stc_therJR0333_FW	CAACCGCATTTGCCACTGCTCCCGAGAAG
stc_therJR0333_RV	CTTCTCGGGAGCAGTGGCAAATGCGGTTG

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