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Promoted evolution of a shortened variant of heme A synthase in the membrane of *Bacillus subtilis*

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Abstract ***Bacillus subtilis*** **heme A synthase is a membrane protein with 8 transmembrane segments. By using a two-step mutagenesis approach we have generated and selected a fully functional enzyme protein variant with a seven residue internal deletion. The biochemical properties of the shortened variant are similar to those of the normal enzyme. This could indicate that residue H209 in the mutant protein substitutes for the missing H216 as an axial ligand to the heme iron. Our results provide insight in routes of membrane protein evolution and the structure of heme A synthases.**

Keywords: CtaA; Heme A synthesis; Membrane protein evolution; Cytochrome *a*; *Bacillus subtilis*

1. Introduction

Evolution of a protein into a stable variant with altered structure and functional properties generally requires several mutations that are introduced stepwise along a so called evolutionary trajectory [1]. One category of such evolution is the emergence of antibiotic resistance in bacteria, where an initial mutation (in, e.g., a protein-coding gene) confers decreased susceptibility to the action of the antibiotic but decreased fitness of the organism. Additional mutations that increase fitness are then accumulated in the same gene or other genes [2]. In this work we have promoted the evolution of an integral membrane-protein that functions in heme A biosynthesis.

Heme A is a prosthetic group of cytochrome *a*, and is, therefore, essential for the function of respiratory oxidases in many organisms. Synthesis of heme A is catalysed by heme A synthase (HAS) which is an integral membrane-bound enzyme [3]. In most organisms, HAS comprises a single polypeptide chain with eight transmembrane segments. A few Archaea, e.g. *Aeropyrum pernix*, have a so called compact variant of the HAS polypeptide. This protein is about half the size, with only four transmembrane segments, and seemingly forms a homo-dimer in the membrane [4]. The HAS of the gram-positive bacterium *Bacillus subtilis* is encoded by the *ctaA* gene [5]. The CtaA protein has eight transmembrane segments (see Fig. 1A) and, as isolated, contains low spin protoheme IX (heme B) but is also sometimes associated with the enzyme product, heme A [6,7]. In previous work we replaced individually four conserved histidine residues of *B. subtilis* CtaA (H60, H123, H216 and H278) with leucine and methionine, in order to analyse the role of these residues [6]. The data suggested that H216 functions as an axial ligand to heme iron. The H216M variant was found to bind heme O (enzyme substrate), probably with methionine as an axial ligand, but was defective in heme A synthesis and accumulated a mono-hydroxylated reaction intermediate, heme I.

Here we have made use of the CtaA-H216M variant to promote the selection of a novel CtaA polypeptide variant in which seven internal residues are deleted, but which is fully functional in heme A synthesis. The results illustrate and explain how an integral membrane protein can evolve via sequential intragenic mutational steps. In addition, our findings lend further support to the proposed functional role of H216 as an axial ligand to heme iron in *B. subtilis* HAS.

2. Materials and methods

2.1. Bacterial strain, plasmids and primers

B. subtilis LMT20R (*trpC2*, *rex-10,* Δ*ctaA::spc*) completely lacks the *ctaA* gene [6]. Plasmids used in this work are listed in Table 1. Oligonucleotides used as primers for amplification of the *ctaA* gene were ctaA1 and ctaAHIA [6] and primers used in DNA sequencing were H123L (5′-GATTATGGCGCTTCTTTTCGGCATCTCATTAATTTC) and CTAA10 (5′-AGAACGCCAAACAGACAG).

2.2. Growth of bacterial cultures

B. subtilis LMT20R containing different plasmids was grown in LB [8] or NSMP (nutrient sporulation medium with phosphate) [9] supplemented with 0.5% glucose and chloramphenicol, 4 μg/mL, or on TBAB (tryptose blood agar base) (Difco) supplemented with antibiotic. Cells for cytochrome *c* oxidation activity analysis were grown in NSMP without glucose. Liquid cultures were grown at 37 °C in 1 L medium in 5 L baffled E-flasks at 200 rpm on a rotary incubator.

2.3. Chemical mutagenesis

An over night culture of *B. subtilis* LMT20R/pH216M was used to inoculate 50 mL LB and the culture was grown at 37 °C to exponential growth phase. The cells were pelleted by centrifugation, suspended in LB and treated with 5% (v/v) ethyl methanesulfonate (EMS) at 30 °C, over night. EMS-treated cells were washed three times using 50 mM potassium phosphate buffer, pH 7.0, and then stored frozen in 25% (v/v) glycerol at −70 °C.

2.4. Miscellaneous techniques

N,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD)-oxidation staining of colonies on TBAB plates was done as described before [10,11]. Plasmids were extracted from cells using the E.N.Z.A plasmid Miniprep kit (Omega Bio-Tek) and *B. subtilis* cells were transformed after being grown to natural competence as described by Hoch [12]. Sequence analysis of the *ctaA* gene in plasmid was done by first

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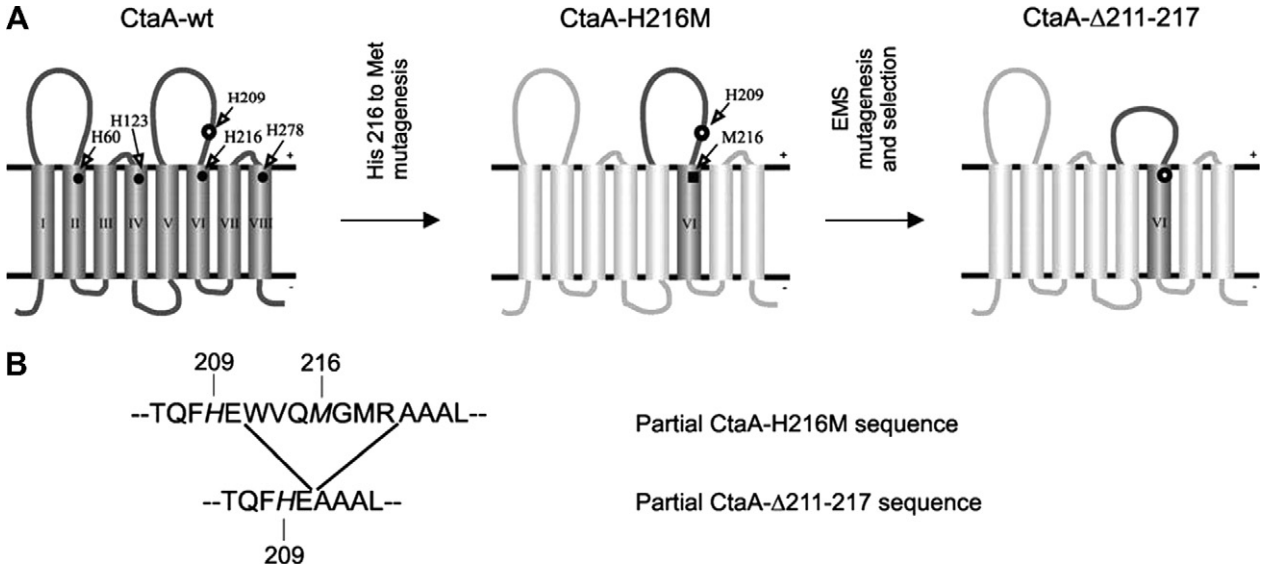

Fig. 1. Evolution of a shortened CtaA polypeptide: (A) Schematic drawing of the *B. subtilis* wild type CtaA (CtaA-wt) and two variants (CtaA-H216M and CtaA-Δ211–217). The variants were obtained in two steps; by site-directed mutagenesis and random mutagenesis and selection, respectively. Transmembrane α-helices are numbered I–VIII. Invariant histidine residues are indicated by black dots and residue number. A not conserved histidine residue, H209, is indicated by a circle. In the CtaA-H216M variant, residue M216 is indicated by a black square. + and − denote outside and inside of the cellular membrane, respectively. (B) Partial amino acid sequence of the CtaA-H216M and CtaA-Δ211–217 variants. The deletion of residues 211–217 is indicated. Residues 209 and 216 are high-lighted.

Table 1
Plasmids used

Plasmid	Properties	Source or reference
pHPKS	Cloning vector; Cam^R, Ery^R	[23]
pCTHI10	pHPKS derivative with wild type *ctaA* under its native promoter and encoding a His_6-tagged CtaA; Cam^R, Ery^R	[6]
pH216M	pCTHI10 derivative encoding CtaA-H216M; Cam^R, Ery^R	[6]
pH216MS	Obtained from pH216M and carries a 21 bp deletion in *ctaA*; Cam^R, Ery^R	This work

amplifying the gene using PCR and primers ctaA1 and ctaAHIA. The PCR product was sequenced at MWG GmbH (Martinsried, Germany) using primers CTAA10 and H123L. Membranes were isolated as described before [13] and used for cytochrome *c* oxidase activity measurements [14], spectral analysis [15] and analysis of heme composition using HPLC [6]. Affinity purification of CtaA, heme extraction from membranes and isolated protein were done as previously described [6]. The redox potential of isolated CtaA-wt and CtaA-Δ211–217 was analysed as before [7], with the exceptions that the heme content of the samples was between 1 and 3 μM, the absorption spectrum was recorded between 500 and 650 nm using an OLIS DW-2 spectrophotometer and the redox potential was measured using an ORP-146 Micro combination redox electrode (LAZAR), which was calibrated using horse heart cytochrome *c* ($E_{m,7}$ = +178 mV) and quinhydrone ($E_{m,7}$ = +286 mV). Redox titrations were repeated three times for each protein variant.

3. Results and discussion

3.1. Chemical mutagenesis of B. subtilis LMT20R/pH216M

B. subtilis LMT20R is deleted for the *ctaA* gene and, therefore, completely blocked in heme A synthesis. Strain LMT20R containing plasmid pCTHI10, which encodes wild-type CtaA with a C-terminal hexa-histidyl tag (added to facilitate purification of the protein), exhibited normal heme A synthesis [6]. Plasmid pH216M is identical to pCTHI10 except that it encodes a CtaA variant in which histidine residue 216 is replaced by methionine. CtaA-H216M produced in LMT20R/pH216M has very low activity [6].

To find mutations in the *ctaA* gene of plasmid pH216M that result in restoration of HAS activity, *B. subtilis* LMT20R/pH216M cells were subjected to mutagenesis using EMS. The three nucleotide substitutions in codon 216 of the *ctaA* gene in pH216M (CAC to ATG) make back reversion to the wild type sequence unlikely. The survival of cells, determined as colony forming units on TBAB plates, was 0.01% after treatment with 5% EMS. Approximately 850 colonies of surviving cells were screened for active HAS using the TMPD-oxidation staining method. TMPD is an artificial electron donor that is oxidised by cytochrome caa_3 in the respiratory chain of *B. subtilis*. TMPD-oxidation staining of colonies therefore provides an indirect measurement of heme A synthesis in the cell. One TMPD-oxidation positive clone was obtained. Plasmid from that clone was extracted and used to transform *B. subtilis* LMT20R to chloramphenicol resistance. The resulting transformants were TMPD-oxidation positive, showing that the mutation in the original clone was plasmid-born. The plasmid of one transformant was kept and denoted pH216MS.

3.2. Intragenic deletion in ctaA

DNA sequence analysis of the entire *ctaA* gene in plasmid pH216MS revealed a 21 bp deletion within the polypeptide-coding region. This results in the loss of seven residues in the predicted loop on the extra-cytoplasmic side of the membrane, connecting transmembrane helices V and VI in CtaA, see Fig. 1A. Notably, the deletion includes residue 216 (Fig. 1B). The shortened CtaA polypeptide is denoted CtaA-Δ211–217.