Knock-out of the *cyaY* gene in *Escherichia coli* does not affect cellular iron content and sensitivity to oxidants

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Abstract Friedreich ataxia is a recessively inherited neurodegenerative disease caused by deficiency of a highly conserved mitochondrial protein, frataxin. Frataxin deficiency results in mitochondrial iron accumulation and oxidative stress. Frataxin shows homology with the *CyaY* proteins of γ-purple bacteria, whose function is unknown. We knocked out the *CyaY* gene in *Escherichia coli* MM383 by homologous recombination and we generated an *E. coli* MM383 strain overexpressing *CyaY*. Bacterial growth, iron content and survival after exposure to H_2O_2 did not differ among these strains, suggesting that, despite structural similarities, *cyaY* proteins in bacteria may have a different function from frataxin homologues in mitochondria.

Key words: Friedreich ataxia; CyaY; Iron; Free radical; Hydrogen peroxide; *Escherichia coli*

1. Introduction

Friedreich ataxia (FRDA) is an autosomal recessive degenerative disease characterized by atrophy of long axonal tracts in the spinal cord, large primary sensory neurons, cardiomyocytes and possibly pancreas β-cells [1]. Patients develop ataxia and hypertrophic cardiomyopathy, and are at high risk for diabetes mellitus [2]. The disease is caused by a deficiency of frataxin, a small mitochondrial protein (210 amino acids, 150 amino acids after mitochondrial import and removal of the N-terminal targeting sequence) encoded by a gene on chromosome 9q13 [3]. Frataxin deficiency is in most cases consequent on homozygosity for an expanded GAA triplet repeat in the first intron of the gene, occasionally on heterozygosity for a GAA expansion and a point mutation [3]. The FRDA gene is highly expressed in mitochondria-rich cells, particularly in those affected by the disease [4,5]. Though not resembling any protein with known function, frataxin shows a remarkable evolutionary conservation, with homologues in mammals, *Caenorhabditis elegans*, and yeast. Knock-out of the yeast homolog gene (*YFH1*) results in a 10-fold increase of mitochondrial iron, increased sensitivity to oxidants, and eventually in loss of mitochondrial DNA and a *petite* phenotype [6–8]. Together with preliminary evidence that iron metabolism is altered in the human disease, including the presence of iron deposits in the myocardium of affected individuals [9], these findings indicate that frataxin has a role in controlling mitochondrial iron homeostasis, probably by stimulating the efflux of iron out of these organelles [10]. The most conserved portion of frataxin also has a significant similarity to the *cyaY* proteins of γ-purple bacteria, whose function is unknown (Fig. 1). In enterobacteria, the *cyaY* gene is localized near the gene encoding adenylyl cyclase (*cya*), hence its name, before the downstream *dapF* gene [11]. Its open reading frame (ORF) is in the complementary strand with respect to *cya*. A −35 to −10 promoter region, similar to σ70 promoters, precedes the *cyaY* gene, followed by a consensus ribosome-binding sequence (Shine-Dalgarno) before the start codon [11]. Therefore, *cyaY* has its own transcription and translation control region, independent of *cya*. Fusion constructs in which the *cyaY* promoter was inserted upstream of the *lacZ* gene permitted expression of a significant amount of β-galactosidase, indicating that this gene is expressed in *Escherichia coli* [11]. The sequence similarity between *cyaY* and frataxin was one of the first clues of the mitochondrial localization of frataxin, as γ-purple bacteria are related to the ancestor of mitochondria [12]. *CyaY* proteins are not found in bacteria thought to be unrelated to the ancestor of mitochondria, such as Gram-positive species, while *Rickettsia prowazeckii*, possibly the closest living species to the ancestor of mitochondria, has the *cyaY* gene with highest similarity to the eukaryotic frataxin homologs [13]. It was proposed that eukaryotic frataxin homologs and *cyaY* proteins form a family of proteins sharing a structural domain which is conserved from bacteria to mammals [12]. In order to investigate whether this conserved structure is also reflected in a conserved function, namely control of intracellular or intramitochondrial iron concentration and sensitivity to oxidative stress, we knocked out the *E. coli cyaY* gene by homologous recombination and evaluated the growth properties, iron content and sensitivity to H_2O_2 of *cyaY*$^-$ bacteria compared to a wild-type and a *cyaY*-overexpressing strain.

2. Materials and methods

2.1. Bacterial strains

E. coli DH5α was used as a temporary host during plasmid construction. *E. coli* MM383 was obtained from the *E. coli* Genetic Stock Center of Yale University, This strain has a temperature-sensitive DNA polymerase I (*polA* gene) and is totally defective in nick translation at 42°C.

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2.2. DNA manipulations and bacterial cultures

Preparation of chromosomal or plasmid DNA from bacteria, bacterial transformations (except when using the MM383 strain), DNA amplification by PCR, electrophoresis, restriction enzyme digestions, DNA ligation, DNA sequencing, were all performed according to standard protocols [14]. Because of their *polA* temperature-sensitive mutation, *E. coli* MM383 were grown at 30°C and heat shocked at 37°C for transformation. LB and minimal medium were prepared according to standard protocols. MM383 and derived strains need the addition of thymine to grow in minimal medium.

2.3. Construction of a cyaY-overexpressing MM383 derivative (Bfh2)

pOSEX4 was used as plasmid expression vector (a kind gift from Dr. E. Bremer). The *cyaY* gene was amplified from *E. coli* chromosomal DNA using the primers cyaY1-F (5′- TTGGATCCT-TAACACAGGAAACAGACCATGAACGACAGTGAATTTCAT-3′) and cyaY2-R (5′-AAAGGTACCTTAGCGGAAACTGACTGT-T-3′), which generate an amplified fragment with a *Bam*HI site at the 5′ end of the gene and a *Kpn*I site at the 3′ end of the gene. The fragment was then ligated into pOSEX4 at the *Bam*HI/*Kpn*I cloning site forming pMP501, which was then used to transform MM383, obtaining the *cyaY*-overexpressing Bfh2 strain.

2.4. Knock-out of cyaY in MM383 by homologous recombination to obtain the Bfh1 strain

CyaY was disrupted by the insertion of a kanamycin resistance gene (*kan*^{r}) essentially as described by Gutterson and Koshland [15]. Two fragments corresponding to sequences flanking the *cyaY* gene were amplified from *E. coli* MM383 chromosomal DNA. The upstream flank, UF, was amplified using the UF1-F (5′-GCACCGTGGAT-TGCGTTTGCGTCT-3′) and UF2-R (5′-TCGCTGTCGACATCC-CAGTCGTCCA-3′) primers. The downstream flank, DF, was amplified using the DF1-F (5′- GGATTTGCTGGAACAGGCGTCGAC-3′) and DF2-R (5′-TTGCGGGAATTCAACCGGCACATCT-3′) primers. These primers contain the underlined restriction sites, *Sal*I for UF2-R, *Eco*RI for DF2-F and DF2-R. UF and DF were digested by *Sal*I, ligated to each other and inserted into *Eco*RI/*Pst*I-digested pUC18, obtaining pMP502. A kanamycin resistance gene (*kan*^{r}) from pUC4K was inserted at the *Sal*I site between UF and DF forming pMP503. The whole UF-*kan*-DF fragment was then excised from pUC18 using *Eco*RI and *Pvu*II and inserted into *Eco*RI/*Msc*I-digested pBR322, obtaining pMP504 (Fig. 1). pMP504 was used to transform MM383 at 30°C and a colony from the above transformant was grown in LB with streptomycin (50 μg/ml) and kanamycin (50 μg/ml) overnight at 42°C to promote plasmid integration. About 200 μl of the above culture were grown in LB with only streptomycin at 30°C for 3–4 h to eliminate episomal plasmids, then the temperature was switched back to 42°C and kanamycin was added to select for cells with an integrated *kan*^{r} gene. An aliquot of the culture was plated on streptomycin, 96 colonies were picked and separately tested for kanamycin and ampicillin resistance. The two colonies resistant to kanamycin but not to ampicillin had an integrated *kan*^{r} gene but no pBR322 sequences, so they were expected to have a disrupted *cyaY* gene (Fig. 2). PCR analysis confirmed that the *cyaY* gene in these cells had been disrupted by the replacement of most of its coding sequence with a *kan*^{r} gene (Fig. 3).

2.5. Iron concentration assays

Bacteria were grown in 20 ml of LB, either with no iron supplement or with a supplement of 10, 30, 100, 1000 or 3000 μg/ml FAC (ferric ammonium citrate, Fisher, USA), at 37°C for about 11 h with vigorous shaking (300 rpm). The cells were washed four times with 1×fresh LB medium, twice with 10 ml and twice with 1 ml, and their OD_{600} was determined. Six iron determinations were obtained for each sample using a Perkin Elmer Optima 3100XL instrument (inductively coupled plasma optical emission spectroscopy) in which each sample was run in duplicate on three dedicated lines. Iron concentrations were expressed as nmol/10^9 cells.

2.6. H_2O_2 sensitivity assays

Bacteria were grown in LB with vigorous shaking (300 rpm) at 37°C to a density of 0.8×10^7 to 2×10^7 cells/ml and then challenged with 0.5 mM H_2O_2 (Sigma) in 1 ml of LB for 10 min. The challenge was terminated by diluting the culture 60 times with LB. For survival studies, cells were plated in top agar and colonies were counted after 24–36 h.

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Human              1  MWTLGRRAVAGLLASPSPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRRASSNQR
Mouse              1  MWAFGGRAAVGLLPR-TASRASAWVGNPRWREPIVTCGRRGLHVTVNAGAT-RHAHLNLH
C.elegans          1  ------------------------------------------------------------
R.prowazeckii      1  ------------------------------------------------------------
S.cerevisiae       1  ----------------------MIKRSLASLVRVSSVMGRRYMIAAAGGERARFCPAVTN
E.coli             1  ------------------------------------------------------------

Human             61  GLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYERL----AEETLDSLAEFFEDL-AD
Mouse             59  YL-QILNIKKQSVCVVHLRNLGTLDNPSSLDETAYERL----AEETLDSLAEFFEDI-AD
C.elegans          1  ----------MLSTILRNNFVRRSFSSR-IFSQNEYETA----ADSTLERLSDYFDQI-AD
R.prowazeckii      1  ----------------------------MNNTAFSKL----AETTIVYIVDKIEEQ-DL
S.cerevisiae      39  KKNHTVNTFQKRFVESSTDGQVVPQEVLNLPLEKYHEE----ADDYLDHLLDSLEELSEA
E.coli             1  ----------------------------MNDSEFHRL----ADQLWLTIEERLDDW-DG

Human            116  KPYTFEDYDVSFGSGVLTVKLGGDLGTYVINKQTPNKQIWLS--SPSSGPKRYDWT----
Mouse            113  KPYTLEDYDVSFGDGVLTIKLGGDLGTYVINKQTPNKQIWLS--SPSSGPKRYDWT----
C.elegans         46  SFPVSEQFDVSHAMGVLTVNVSKSVGTYVINKQSPNKQIWLS--SPMSGPKRYDLEE----
R.prowazeckii     27  EGIIDVDLQG------DILNLDTENGIYVINTQSASKEIWLS--SPVSGPHHFFYEQ----
S.cerevisiae      95  HPDCIPDVELSHG---VMTLEIPAFGTYVINKQPPNKQIWLA--SPLSGPNRFDLLN----
E.coli            27  DSDIDCEING--G---VLTITFENGSKIIINRQEPLHQVWLA--TK-QGGYHFDLK----

Human            170  -G----------------KNWVFS-HDG----VSLHE-----------LLAAELTKAL
Mouse            167  -G----------------KNWVYS-HDG----VSLHE-----------LLARELTKAL
C.elegans        101  EGKLIYVKQRAVRYFGVSLDNQIFS-HNFQNENFTISERDSSPSTTKKSFLLGALLLFKQ
R.prowazeckii     76  -G------------------KWKS--RIG----------------F-----ELMVLLTEEL
S.cerevisiae     147  -G------------------EWVSL-RNG--------------------TKLTDILTEEV
E.coli            75  -G----------------DEWICD-RSG--------------------ETFWDLLEQA

Human            195  KTKLDLSWLAYSGKDA-
Mouse            192  NTKLDLSSLAYSGKGT-
C.elegans        160  MPRLALRQYNFYYRVNG
R.prowazeckii     95  GIRFDKYEIF-------
S.cerevisiae     167  EKAISKSQ----------
E.coli            95  ATQQAGETVSFR-----
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Fig. 1. Multiple sequence alignment (ClustalW 1.74) of human, mouse, *C. elegans*, *Saccharomyces cerevisiae* frataxin homologs and *E. coli* and *R. prowazeckii cyaY* proteins. Homology between the eukaryotic and the prokaryotic proteins begins after the N-terminal portion of the eukaryotic proteins containing the mitochondrial targeting sequence.

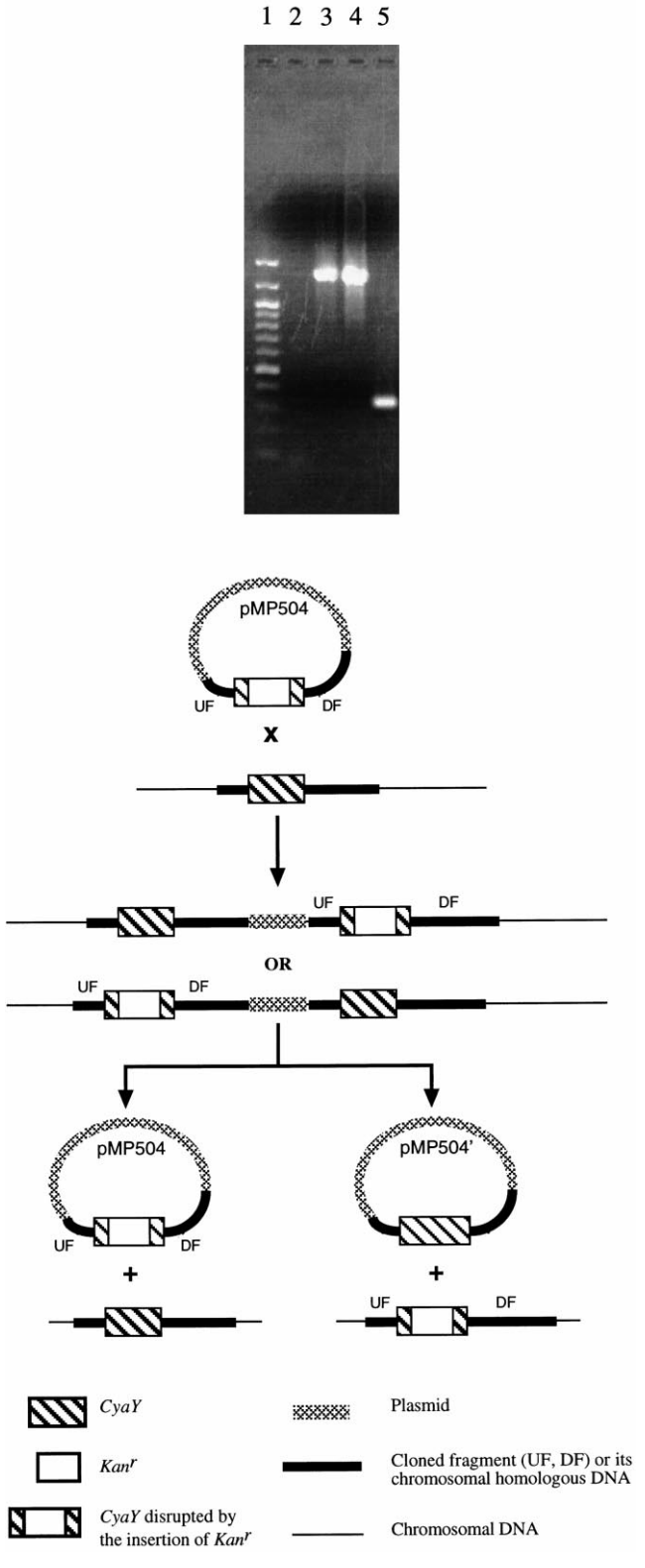

Fig. 2. Upper panel: PCR analysis showing a disrupted *cyaY* gene in Bfh1. The *cyaY* gene was amplified by PCR using the cyaY1-F and cyay2-R primers. In wild-type MM383 (lane 5) the resulting fragment has a size of 0.33 kb; in Bfh1 (lanes 3 and 4) the fragment has a size of 1.35 kb because of the insertion of the *Kan*^{r} gene (1.25 kb) and the loss of *cyaY* sequence. Lane 1: molecular size markers (1 kb ladder); lane 2: blank. Lower panel: Scheme of the replacement of the segment of chromosomal DNA containing the *cyaY* gene by the homologous sequence in the plasmid.

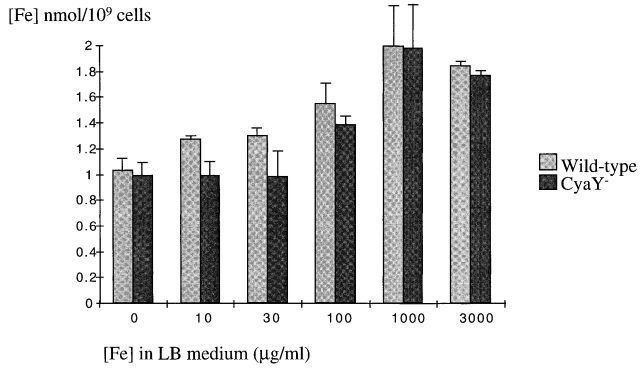

Fig. 3. Intracellular iron in wild-type MM383 and Bhf1 *E. coli* after 11 h of growth in LB medium supplemented with the indicated amounts of iron (as ferric ammonium citrate).

3. Results and discussion

The *cyaY* gene of *E. coli* MM383 was disrupted by substituting most of its open reading frame with a *kan*^{r} gene. The temperature-sensitive *polA* mutation of MM383 was exploited to insert a pBR322-derived plasmid carrying a disrupted *cyaY* gene into chromosomal DNA by homologous recombination, then to excise the plasmid leaving a disrupted chromosomal copy of *cyaY* (Fig. 2). Δ*cyaY* bacteria (Bfh1) were selected for kanamycin resistance and ampicillin sensitivity. Conversely, a *cyaY*-overexpressing strain (Bfh2) was obtained by cloning the *cyaY* open reading frame into the pOSEX4 expression vector and using the resulting pMP501 plasmid to transform MM383. Wild-type MM383, Bfh1 and Bfh2 did not show differential growth either in LB or in minimal medium+thymine, and formed colonies of identical morphology. Wild-type and Bfh1 cells were grown for 11 h in LB medium supplemented with various amounts of iron (as FAC), in which they grew equally well. When iron content was determined by inductively coupled plasma optical emission spectroscopy, it was found not to differ between the two strains, which were both able to maintain iron homeostasis (Fig. 3). Exposure to a low dose of H_2O_2 equally reduced the survival of wild-type MM383 and of Bfh1 and Bfh2 (Fig. 4).

From the above results, it can be concluded that, at least in the tested conditions, *cyaY* affects neither intracellular iron

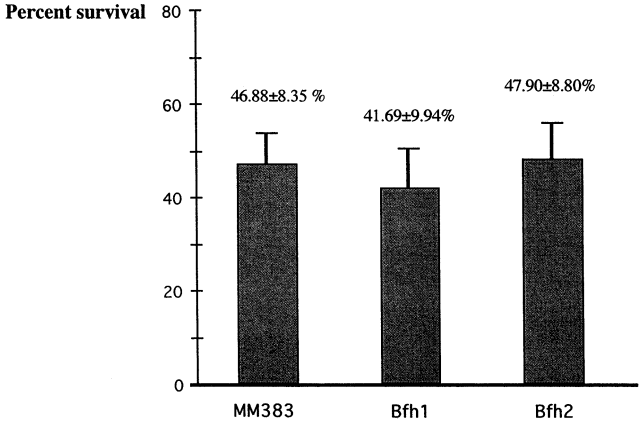

Fig. 4. Percent survival of wild-type MM383, Bhf1 and Bhf2 *E. coli* after a 10 min exposure to 0.5 mM H_2O_2.

content nor resistance to exogenous oxidants such as H_2O_2. Considering that no difference in growth rate or in colony morphology was found between wild-type, Δ*cyaY*$^-$ and *cyaY*-overexpressing bacteria, either in rich medium (LB) or in minimal medium, the function of this protein remains obscure. Consequently, a disappointing aspect of our findings is that it does not seem possible to exploit the simple genetics of bacteria to obtain data relevant to frataxin function.

Structurally, the similarity between *cyaY* proteins and the mature intramitochondrial forms of eukaryotic frataxin homologs remains remarkable. The similarity in amino acid sequence is highly significant, and the predicted structure is essentially the same: a central β-sheet region spanning the most conserved domain, flanked by two α-helices [12]. However, the difference of function between *cyaY* and the eukaryotic frataxin homologs does not come completely as a surprise, because there are many examples of proteins with an overall conserved structure but a modified function during evolution. The small size of both mature frataxin and *cyaY* makes it unlikely that these proteins have enzymatic activity. Accordingly, it has been proposed that they may act as regulators or metabolite carriers [12]. The yeast frataxin homolog (yfh1p) is likely to stimulate the outflow of non-heme iron from mitochondria [10], a function that is fully compatible with a regulator or metabolite carrier role. Bacterial *cyaY* proteins may have a similar function, except that the carrier system they activate or the metabolite they bind does not affect iron homeostasis. It is somewhat surprising that *cyaY* knockout has no evident effect on bacterial viability, considering how conserved *cyaY* proteins are among purple bacteria. The fact that a *cyaY* gene is present in *R. prowazeckii* [13] despite the reduction that the genome of this intracellular parasite has undergone would point to a more essential function. Perhaps this gene has acquired a new function with the passage to intracellular life, related to metabolic exchanges with the host cell, as has happened for the mitochondria precursor.

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