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A new homolog of FocA transporters identified in cadmium-resistant *Euglena gracilis*

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Abstract

To better understand the cellular mechanism of stress resistance to various pollutants (cadmium, pentachlorophenol), we undertook a survey of the *Euglena gracilis* transcriptome by mRNA differential display and cDNA cloning. We performed a real-time RT-PCR analysis upon four selected genes. One of them significantly changed its expression level in response to stress treatments: B25 gene was overexpressed in Cd-resistant cells whereas it was down-regulated in PCP-adapted cells. By Race assays we obtained for B25 a 1093 bp cDNA. The deduced protein was identified as a bacterial formate/nitrite transporter (FocA) homolog and the gene was named *EgFth*. From all the data, we concluded that *EgFth* overexpression was related to chronic exposure to cadmium.

Keywords: *Euglena gracilis*; Xenobiotics; Cadmium; Resistance; Gene overexpression

Heavy metal pollution is a worldwide problem and cadmium (Cd) is one of the most noxious environmental pollutants responsible for episodes of chronic poisoning in humans [1,2]. We focus our interest on the resistance response of *Euglena gracilis* to cadmium and to other fresh water pollutants such as the organochlorated compound pentachlorophenol (PCP) to better understand mechanisms of cell resistance and to characterize new eukaryotic molecular markers of adaptation to xenobiotics.

Euglena gracilis, a fresh water protozoon, is one of the eukaryotic models used to study Cd-induced toxicity and related areas. *Euglena* exhibits plant-like and animal-like characteristics [3], and possesses well-conserved proteins [4,5]. Its attractiveness as a model organism is exemplified by its continued extensive use for toxicology and bioremediation research [6–12].

Using available probes or antibodies against ubiquitous stress-related genes, we previously reported the overexpression of heat-shock proteins in Cd-resistant and PCP-adapted *E. gracilis* strains [13,14]. We also reported the constitutive overexpression of CYP-450 related proteins in PCP-adapted *E. gracilis* [15]. The purpose of the present study was to investigate the involvement of genes yet unknown for their role in xenobiotic-resistance mechanisms. Comparison of the transcriptional pattern of Cd-resistant *E. gracilis* cells with PCP-adapted or control ones was performed using mRNA differential display. Candidate genes were cloned and their expression level was evaluated. We show here that Cd-resistant *Euglena* cells overexpress *EgFth*, a new gene encoding a putative bacterial FocA transporter homolog.

Materials and methods

Cell cultures. Axenic cultures of achlorophyllous *Euglena gracilis* W100ZUL cells were grown at 23 °C in mineral medium supplemented

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with vitamins B1 and B12 and containing lactic acid (33 mM) as the carbon source [16]. From this strain, a cadmium-resistant cell line was established [17] by progressively increasing the Cd concentration from 500 μM to 2 mM in the culture medium. Cd-resistant cells have been subcultured with 2 mM Cd for 15 years (RM) or without Cd for 10 years (RwoM). A PCP (pentachlorophenol)-tolerant cell line was also established and subcultured with 4 μM PCP for 12 years (RP) or without PCP for 5 years (RwoP) [17]. We also established a cell line resistant both to 2 mM Cd and 4 μM PCP, which was subcultured for 10 years with both xenobiotics (RM+P). All cell lines were analysed from cultures to mid-logarithmic growth phase, i.e. after 2–3 days of culture.

RNA preparation. Total RNA was extracted either from 10^6 cells using the high Pure RNA Isolation kit (Boehringer–Mannheim), or from 4×10^7 cells using the original guanidinium thiocyanate-phenol method [18]. Quality control of total RNA was performed either by denaturing agarose gel electrophoresis or by the Bioanalyzer 2100 (Agilent Technologies) technology. Messenger RNA isolation was carried out from 10^8 cells on oligo-dT affinity columns, using the Fast Track™ 2.0 kit (Invitrogen) according to the supplier's instructions.

mRNA differential display. The differential display procedure was adapted from the protocol described in the Delta™ Differential Display kit User's Manual (Clontech). Two μg of total RNA were reverse-transcribed with M-MLV reverse transcriptase (Life Technologies), using the anchored primer 5′-CATTATGCTGAGTGATATCTTTTTTTTTTAA-3′. PCR amplification of the cDNA was performed in the presence of [α-^{33}P]dATP, with the matching downstream primer and P1 (5′-ATTAAC CCTCACTAAATGCTGGGGA-3′), P2 (5′-ATTAACCCTCACTAAAT CGGTCATAG-3′) or P3 (5′-ATTAACCCTCACTAAATGCTGGG GG-3′) as the arbitrary upstream primer, *AmpliTaq*® DNA polymerase (Perkin-Elmer), with hybridization step at 40 °C (first 3 cycles) and 60 °C (30 last cycles) in a thermocycler 480 (Perkin-Elmer). The cDNA fragments that were differentially displayed between cell lines were recovered by elution from the corresponding gel segment in deionized water for 24 h at room temperature. The DNA was re-amplified using the same set of primers as that used to generate the amplicon from cDNA, using 30 cycles as follows: 94 °C for 1 min, 60 °C for 1 min, 68 °C for 2 min. The PCR fragments were cloned into the PCR®2.1 or PCR®4-TOPO™ vector using the TOPO™TA cloning® kit (Invitrogen) and sequenced with an automated DNA sequencer (ABI 310®, Perkin-Elmer) using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). DNA sequences were analyzed by the FASTA or BLAST search programs.

Real-time PCR analysis. First strand cDNA was generated from 2 μg of total RNA using oligo-dT(16) primer (Perkin-Elmer) and M-MLV reverse transcriptase (Life Technologies) according to the manufacturer's instructions, in a total reaction volume of 20 μl. Specific oligonucleotide primers (Table 1) were designed using Primer3 software (http://frodo.wi.mit.educgi-binprimer3/primer3_www.cgi) for sequences of interest and

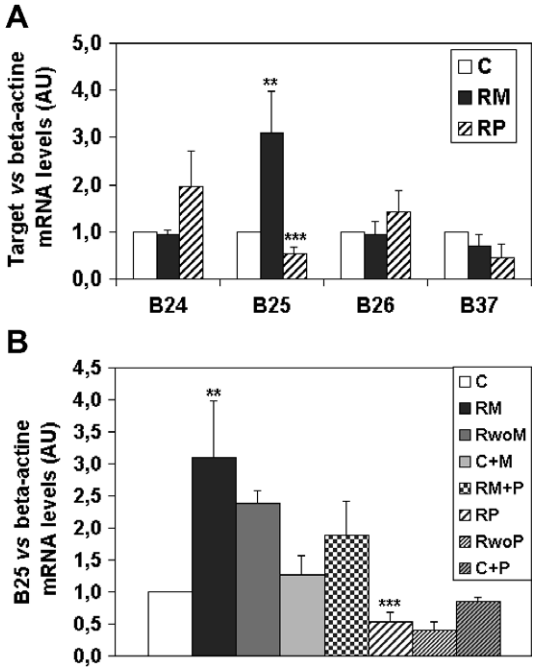

Fig. 1. Real-time PCR analysis. (A) Expression of four cDNA identified by differential display in Cd-resistant *E. gracilis* cells. C: control; RM: Cd-resistant cells grown with 2 mM Cd; RP: PCP-adapted cells grown with 4 μM PCP. B24, B25, B26, B37: designation of the cDNA studied. The results were normalized to the β-actin transcript levels. Statistics by Student's *t*-test: $^{**}p < 0.01$; $^{***}p < 0.002$. A.U.: arbitrary units. (B) Expression of B25 cDNA in *E. gracilis* in mid-log of growth ; C: control; RM: Cd-resistant cells always grown with 2 mM Cd; RwoM: RM cells grown without Cd for 10 years; RM+P: cells resistant both to 2 mM Cd and to 4 μM PCP; RP: PCP-adapted cells always grown with 4 μM PCP; RwoP: RP cells grown without PCP for 5 years; C+M: control cells treated with 2 mM Cd; C+P: control treated with 4 μM PCP. The results were normalized to the β-actin transcript levels. Statistics by Student's *t*-test; $^{**}p < 0.01$; $^{***}p < 0.002$. A.U.: arbitrary units.

Table 1
Oligonucleotides used in this study

Gene[a]	Name	Sequence (5′–3′)	Amplicon size (pb)	Application
Actin	A3	(F) ATG AAT CCC AAG AGC AAT CG	432	QRT-PCR
(AF057161)	A4	(R) GTT TCC ATC AGG CAA CTC GT		
GADPH	GADPH1	(F) GGT GCC AAG AAG GTC ATC AT	150	QRT-PCR
(L21903)	GADPH2	(R) CAC GTG AAC AAG AGG AGC AA		
B24 (DD)	PRO3	(F) CTA AAT GCT GGG GAT GGC GGA CAA	357	QRT-PCR
	PRO4	(R) CAG CCT CAC AGA GCG AGG AAC GAT G		
B25 (DD)	FIB1	(F) AGC CAA CAG CAT TGG AAG TC	221	QRT-PCR
	FIB2	(R) CCA AAG CAG ATC GAT GAC AA		
B26 (DD)	HU1	(F) AAG GAC GAG AAC ATC GTT GC	280	QRT-PCR
	HU2	(R) CCA ATG GGT CAA GGT CTT T		
B37 (DD)	Lae1	(F) GCC AGT TTC TGT TGC AGT CA	162	QRT-PCR
	Lae2	(R) ATG GGG CGT TGT TTT AGA TG		
B25 (DD)	Ra1	(F) TGC TGC CCC GAA TAC TCC CGA TGT CC		Race-PCR
	Ra2	(F) TAC TCT GGG AAC AAT GCT GCC CCG		Race-PCR
Race-PCR product	Ra3	(F) TTT TTG GGA TGT GCG TTG TA	242	RT-PCR
	Ra4	(R) AAC CAG CAA GCA ATC ACC TT		

[a] The name of the gene and its GeneBank accession no. or identification mode; DD: differential display.