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Epigallocatechin-3-*O*-gallate, the main green tea component, is toxic to *Saccharomyces cerevisiae* cells lacking the Fet3/Ftr1

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ABSTRACT

Epigallocatechin-3-*O*-gallate (EGCG), the main green tea component, is intensively studied for its anti-oxidant, anti-inflammatory, anti-microbial and anti-cancer effects. In the present study, a screen on a *Saccharomyces cerevisiae* gene deletion library was performed to identify conditions under which EGCG had deleterious rather than beneficial effects. Two genes were identified whose deletion resulted in sensitivity to EGCG: *FET3* and *FTR1*, encoding the components of the Fet3/Ftr1 high-affinity iron uptake system, also involved in Cu(I)/Cu(II) balance on the surface of yeast cells. The presence of EGCG in the growth medium induced the production of Cu (I), with deleterious effects on *fet3Δ* and *ftr1Δ* cells. Additionally, when combined, physiological surpluses of Cu (II) and EGCG acted in synergy not only against *fet3Δ* and *ftr1Δ*, but also against wild type cells, by generating surplus Cu(I) in the growth medium. The results imply that caution should be taken when combining EGCG-rich beverages/nutraceuticals with copper-rich foods.

1. Introduction

Green tea is one of the most consumed beverages in the world and its health benefits cover a wide range of chemo-protective actions attributed to its chemical constituents, which exhibit various biological and pharmacological properties (Butt, Ahmad, Sultan, Qayyum, & Naz, 2015; Cao, Han, Xiao, Qiao, & Han, 2016; Hayat, Iqbal, Malik, Bilal, & Mushtaq, 2015; Jacob, Khan, & Lee, 2017; Khan & Mukhtar, 2013; Reygaert, 2014; Singhal, Raj, Gupta, & Singh, 2017; Vuong, 2014; Xu, Xu, & Zheng, 2017). The main compounds responsible for the activities of green tea are the polyphenols known as flavanols, with epigallocatechin-3-*O*-gallate (EGCG) being the most abundant (Hara, 2001). Numerous studies indicate that EGCG is a potent antioxidant and anti-inflammatory agent responsible for many of the biological actions of green tea associated with the prevention and/or treatment of chronic diseases, such as cancer, heart diseases, obesity, diabetes and neurodegenerative diseases (Chikara et al., 2018; Eng, Thanikachalam, & Ramamurthy, 2018; Fujiki, Sueoka, Rawangkan, & Suganuma, 2017). In spite of the numerous health benefits, some studies focus on the potential toxicity of excessive consumption of green tea and especially of nutraceuticals containing EGCG, in both human and experimental animals (Dekant, Fujii, Shibata, Morita, & Shimotoyodome, 2017; Rasheed, Ahmed, Abdallah, & El-Sayeh, 2017). Studies concerning the beneficial role of green tea and its components are numerous, but reports on their toxicity are still scarce. In this study attempts were made to unravel the molecular aspects related to EGCG toxicity using a yeast-based chemogenomic screen of a *Saccharomyces cerevisiae* gene deletion library. Yeast chemogenomic approaches can be used to identify molecular components indicative of a cell response upon exposure to various molecules, by testing the fitness of yeast deletion libraries, also known as Yeast KnockOut (YKO) collections (Hillenmeyer et al., 2008). By screening a collection of ~4800 YKO mutants against EGCG exposure it was revealed that cells with no functional Fet3/Ftr1 complex exhibited poor growth in the presence of EGCG, a phenotype that was augmented by surplus copper. Copper is essential for life and a variety of enzymes require copper as a cofactor necessary for electron transfer reactions (De Freitas et al., 2003). Copper in excess is very toxic due to its ability to produce free radicals when cycling between oxidized Cu(II)

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and reduced Cu(I) (Shi, Stoj, Romeo, Kosman, & Zhu, 2003). Copper uptake, buffering and traffic in *S. cerevisiae* have been extensively studied and reviewed (Nevitt, Ohrvik, & Thiele, 2012). Cu(II) ions are taken up from the environment by the cell surface Fet3/Ftr1, which is a high affinity iron uptake system. Fet3 is an oxidase responsible for the oxidation of Fe(II) or Cu(I) to Fe(III) or Cu(II), respectively. The products of Fet3-mediated oxidation, Fe(III) or Cu(II) ions, are subsequently transported into the cell via the trans-membrane permease Ftr1 (Shi et al., 2003). For cell-surface targeting, the Fet3p/Ftr1p complex must be assembled in the endoplasmic reticulum: without Fet3p, Ftr1p is retained in the ER; without Ftr1p, Fet3p is misglycosylated in Golgi apparatus and recycled back to the ER (Sato, Sato, & Nakano, 2004; Stearman, Yuan, Yamaguchi-Iwai, Klausner, & Dancis, 1996; Wang, Quintanar, Severance, Solomon, & Kosman, 2003).

In this study we attempted to unravel molecular aspects related to EGCG toxicity by using a yeast-based chemogenomic screen of a *Saccharomyces cerevisiae* gene deletion library. It was found that cells devoid of either of the Fet3/Ftr1 components develop sensitivity to EGCG. Evidence is provided that this phenotype is related to the surplus Cu(I) produced in the growth medium as result of the antioxidant traits of EGCG.

2. Materials and methods

2.1. Reagents and growth media

Unless otherwise specified, all reagents used were purchased from Sigma-Aldrich (St. Louis, USA). Yeast strains were grown and manipulated as described (Sherman, 2002). Rich YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) or synthetic complete SC (0.67% w/v yeast nitrogen base with $(NH_4)_2SO_4$, 2% w/v glucose, supplemented with the necessary amino acids) were used throughout the study. For solid media, 2% w/v agar was used. All supplementary chemicals were added to the media from sterile concentrate stocks. The aqueous EGCG stock solution (10 mg/ml) was sterilized by filtration (0.22 μm pore size, Millipore, USA), aliquoted and stored at −45 °C in brown tubes to avoid excessive light exposure during manipulation. Stock solution of CuCl (500 μM) was freshly prepared in nitrogen-purged 10 mM 2-(N-morpholino)ethanesulfonic acid- Tris(hydroxymethyl)aminomethane (MES/Tris buffer), pH 6 (Stoj, Augustine, Solomon, & Kosman, 2007).

2.2. Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study were the "wild-type" (WT) parental strain BY4741: *MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0* (Brachmann et al., 1998). The YKO haploid library contained ~4800 strains where each non-essential open reading frame (ORF) was individually replaced with a G418 (geneticin) resistance gene; every YKO strain from the collection (generically named *orfΔ*) had the genotype *MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3*Δ0; *orf::kanMX4*. The strains were obtained from EUROSCARF (European *S. cerevisiae* Archive for Functional Analysis, Institute of Molecular Biosciences Johann Wolfgang Goethe-University Frankfurt, Germany, www.euroscarf.de) and strored at −85 °C as glycerol stocks in 96-well plates, each well containing one individual strain. The YKO collection was recovered before use by replica plating in 150 μl YPD using a sterile 96-pin replicator (Sigma-Aldrich), followed by 1 day incubation at 30 °C.

2.3. Screening and scoring

The restored strains from the YKO collection were transferred at a density of approximately 1×10^5 cells/well (5×10^5 cells/ml) to 96-well plates. Each well had 200 μl SC containing or not (control) 10 μg/ml EGCG. The plates were incubated in a dark room for 24 h at 30 °C, after which they were vortexed and the turbidity of the cell suspensions was determined spectrophotometrically at 600 nm (OD_{600}) using a plate reader (Varioskan, Thermo Fischer Scientific, Vantaa, Finland). The growth index of each strain was calculated as the ratio between OD_{600}-s, determined in the presence and absence of EGCG to identify the EGCG sensitive mutants. Strains with growth ratio less than 0.75 were considered sensitive to EGCG and they were selected for further investigation.

2.4. Growth assessment of selected strains

2.4.1. Growth in liquid media

Overnight pre-cultures were inoculated in fresh SC medium at density 2×10^5 cells/ml. Cells were incubated for two hours with shaking (200 rpm) at 30 °C in a multi-amplitude orbital constant temperature shaking incubator (Shanghai ZHICHENG Analytical Instruments Manufacturing Co, Ltd., China) prior to various tests. The cell density in liquid media was monitored at 2 h-time intervals by determining the turbidity of the cellular suspension at 660 nm (Amberg, Burke, & Strathern, 2005).

2.4.2. Halo assay

The halo assay was performed on SC soft agar plates (Shitamukai, Mizunuma, Hirata, Takahashi, & Miyakawa, 2000). The indicator cells were suspended at a cell density of 1×10^5 cells/ml in molten soft agar SC (0.7% agar, 50 °C), then spread on SC plates (2% agar). After the solidification of the soft agar, the tested compounds were placed onto the surface and incubated at 30 °C. The halo of the growth zone on/around the spotted sample was observed after 3 days incubation at 30 °C.

2.4.3. Cell viability

The cell viability, expressed as percentage of live cells within a whole population, was assessed by staining with methylene blue. Viability was examined for at least 300 cells from one biological replicate (Kwolek-Mirek & Zadrag-Tecza, 2014). Viable cells were colorless, and dead cells were blue. Original cell suspensions (grown in normal medium) had a viability higher than 99%.

2.5. EGCG assay

EGCG quantitation in an aqueous solution was performed using Folin-Ciocalteu reagent (Singleton, Orthofer, & Lamuela-Raventos, 1999). A sample aliquot of 100 μl prepared as described above was mixed with Folin-Ciocalteu reagent (50 μl) in ultra-pure water (0.5 ml) and vortexed. An aqueous solution of Na_2CO_3 (200 g/l, 0.6 ml) was added and the mixture was stored for 30 min in a dark room at ambient temperature before the absorbance was measured at 760 nm on an UV–Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan). To determine EGCG in the growth media, cells were removed by centrifugation and EGCG was determined in the supernatant. EGCG was determined using a callibration curve of EGCG standard solutions in the range 1–20 μg/ml. To determine EGCG uptake by yeast cells, approximately 10^9 cells (pre-washed 2 times) were re-suspended in 100 μl 10% Triton-X100 in 10 mM MES-Tris, pH 6, and boiled for 2 min. After centrifugation (10000 rpm, 1 min), the supernatant was diluted 10 times and used for EGCG assay. Cell EGCG was normalized as milli-equivalents EGCG/10^8 cells.

2.6. Cu(I) assay

Cu(I) was determined spectrophotometrically with the Cu(I)-specific chelator bathocuproine disulfonic acid, disodium salt (BCS). A 50 μM BCS in 10 mM MES-Tris (pH 6) was freshly prepared and mixed (v/v) with the Cu(I)-containing sample. The Cu(I)-BCS complex was monitored at 480 nm on a UV–Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan). A callibration curve was established for Cu(I)