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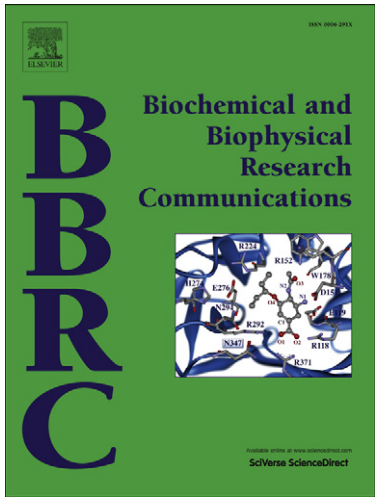

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Tellurite-exposed *Escherichia coli* exhibits increased intracellular α-ketoglutarate

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Abstract

The tellurium oxyanion tellurite is toxic to most organisms because of its ability to generate oxidative stress. However, the detailed mechanism(s) how this toxicant interferes with cellular processes have yet to be fully understood. As part of our effort to decipher the molecular interactions of tellurite with living systems, we have evaluated the global metabolism of α-ketoglutarate a known anti-oxidant in *Escherichia coli.* Tellurite-exposed cells displayed reduced activity of the KG dehydrogenase complex (KGDHc), resulting in increased intracellular KG content. This complex's reduced activity seems to be due to decreased transcription in the stressed cells of *sucA*, a gene that encodes the E1 component of KGDHc. Furthermore, it was demonstrated that the increase in total reactive oxygen species and superoxide observed upon tellurite exposure was more evident in wild type cells than in *E. coli* with impaired KGDHc activity. These results indicate that KG may be playing a pivotal role in combating tellurite-mediated oxidative damage.

Key words: tellurite; α-ketoglutarate; superoxide; reactive oxygen species; oxidative stress

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Introduction

Tellurium is a metalloid belonging to the VIA group of the periodic table of elements that shares various chemical properties with biologically important elements such as oxygen, sulfur, and selenium [1]. Although in its elemental form, Te°, tellurium is rather scarce in nature, the soluble oxyanions tellurite (TeO_3^{-2}) and tellurate (TeO_4^{-2}) are highly toxic to both prokaryotic and eukaryotic cells [2]. Experimental evidence accumulated during the last few years suggests that tellurite toxicity is due, at least in part, to the generation of reactive oxygen species (ROS) [3, 4, 5, 6]. Tellurite-mediated ROS generation was first suggested in studies showing that tellurite minimal inhibitory concentrations (MIC) were higher for tellurite-hypersensitive *Escherichia coli sodAsodB* under anaerobic conditions [7]. The leading ROS generated as consequence of tellurite exposure was shown to be superoxide (O_2^-) [4]. In fact, it was later shown that this radical, produced concomitantly with tellurite reduction, was responsible for the abolition of fumarase and aconitase activity in tellurite-exposed *E. coli* [8].

On the other hand, studies using extracts from *Aeromonas caviae* ST and *E. coli* previously grown in the presence of increasing K_2TeO_3 concentrations showed that while pyruvate dehydrogenase (PDH) activity decreased by ~40%, tellurite reductase (TR) activity increased almost 2-fold. Only dihydrolipoamide dehydrogenase (E3 component of PDH) was shown to display TR activity *in vitro* [9]. Because E3 also forms part of the α-ketoglutarate dehydrogenase complex (KGDHc) [10] tellurite might also affect the normal functioning of this complex, thus

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representing another intracellular target of the toxicant [9]. In this context, a perturbation of various key TCA cycle enzymes activity leading to α-ketoglutarate (KG) accumulation has been observed in *Pseudomonas fluorescens* and eukaryotic HepG2 cells exposed to ROS-generating compounds [11].

KG, an intermediary α-keto acid of the TCA cycle, can detoxify H_2O_2 and O_2^- through spontaneous decarboxylation to yield succinate [12]. It has been proposed that by decreasing KGDHc and increasing isocitrate dehydrogenase (ICDH) activities, cells seem to dedicate some KG to ROS-scavenging with the concomitant drop in NADH biogenesis. Thus, reducing KGDHc activity seems to be pivotal in TCA cycle regulation [11]. In this context, KGDHc activity modulation could play a key role in the cell's ROS-detoxifying strategy.

In this work, we evaluated KG's participation in facing tellurite-induced oxidative stress in *E. coli*. KGDHc activity and KG content was examined in tellurite-exposed *E. coli*. Tellurite sensitivity and ROS content was assessed in wild type and *E. coli* cells lacking *sucA*, *sucB* or *lpdA*, encoding E1 (ketoglutarate dehydrogenase), E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoyl dehydrogenase) KGDHc components, respectively. Results showed decreased KGDHc activity and increased KG content in toxicant-treated bacteria, suggesting that KG accumulation may represent a strategy to cope with tellurite-mediated oxidative damage in *E. coli*. Reduced KGDHc activity seems to result from decreased *sucA* transcription in stressed cells. Finally, it was observed that *E. coli* exhibiting impaired KGDHc activity generates less ROS than the wild type strain.

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Materials and Methods

Bacteria and growth conditions

Bacterial strains used in this work are listed in Table 1. Cells were routinely grown in Luria-Bertani (LB) medium at 37 ^{0}C with shaking. Growth was started by inoculating 1:100 dilutions of overnight cultures in fresh medium. When required, kanamycin (100 µg/ml) was amended to the medium.

Growth inhibition zone (GIZ) and minimal inhibitory concentration (MIC) determination

GIZ were determined as described earlier [13]. In brief, cultures were grown to OD_{600} ~0.5 and 100 µl aliquots were evenly spread on LB-agar plates. After air drying, 10 µl of K_2TeO_3 10,000 µg/ml were deposited on sterile filter disks (6 mm) previously placed on the plate centers. Growth inhibition zones were determined after overnight incubation at 37 ^{0}C.

MICs were assessed as follows. Sterile stock solutions of appropriate K_2TeO_3 concentrations were serially diluted in 96-well ELISA plates containing 200 µl of LB medium amended with the appropriate antibiotic per well. Cultures (5 µl) grown to OD_{600} ~0.5 were added to each well and plates were incubated at 37 ^{0}C. Turbidity was observed visually after 24 h.

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Assessment of intracellular KG and succinate content

KG levels were determined by high-performance liquid chromatography (HPLC). Cells *E. coli* BW25113 (wild type) were grown in LB medium and exposed to 0.5 µg/ml tellurite for 5, 15 or 30 min. After centrifuging at 8,000 x *g* for 3 min, cells were washed and suspended in 350 µl of 25 mM phosphate buffer pH 7.0. After sonication, the cell debris was discarded and supernatants were treated with 200 µl of 0.5% (v/v) perchloric acid and centrifuged and filtered before injection into an Alliance HPLC (Waters) apparatus equipped with an Agilent Hi-Plex H (300 x 7.7 mm) column operating at a flow rate of 0.6 ml/min. Runs lasted 30 min. KG and succinate were detected at 210 nm using a dual wavelength absorbance detector. The mobile phase was 5 mM H_2SO_4 at 55 °C. Metabolites were identified by comparison with known standards and bands were quantified using the Empower software (Waters Corporation). Protein concentration was normalized as described earlier [14].

qRT-PCR

Cells grown in LB medium to OD_{600} ~0.5 were exposed to 0.5 µg/ml tellurite for 30 min. Total RNA was purified using the RNAsy kit (Qiagen), as recommended. Two µg of purified RNA was used as template for qRT-PCR. Reactions were performed using the LightCycler RNA Amplification SYBR Green I kit (Roche Applied Science) as recommended by the vendor. Transcript amounts

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(ng) of *sucA, sucB and lpdA* mRNA were calculated from a standard curve made with known template concentrations. Specific primers used to amplify the genes under study are indicated in Table 1. *rpoD* was used to normalize the experiment.

ROS monitoring by flow cytometry

To determine total ROS, *E. coli* BW25113 and KGDHc mutants were grown to OD_{600} ~0.5 and exposed to 0.05 µg/ml tellurite for 30 min. After washing, centrifuging and suspending in 500 µl of 25 mM phosphate buffer pH 7.0 (buffer A), cells were incubated with 0.02 mM 2,7 dihydrodichlorofluorescein diacetate (H_2CFDA, final concentration) for 30 min in the dark. Fluorescence intensity was monitored as above (λ_{ex} 428, λ_{em} 522). Data acquisition was performed by counting the number of positive cells as recently described [15]. Cells exposed to 5 mM TBH (tert-butyl hydroperoxide) were used as positive control for total ROS detection.

To assess superoxide, *E. coli* BW25113 and KGDHc mutants strains were grown to OD_{600} ~0.5 and exposed to 0.05 µg/ml tellurite for 30 min. After centrifuging and washing with buffer A, cells were suspended in 500 µl of buffer A and incubated with 0.05 mM dihydroethidine (DHE, final concentration) for 15 min in the dark. Intensity was assessed using a Becton Dickinson (model FacsCanto II) apparatus equipped with an Argon laser (λ_{ex} 520, λ_{em} 610). Tellurite-exposed Δ*sodAsodB E. coli* was used as tool for oxidized DHE detection.

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KGDHc, complex I and NADH oxidase activity determination

KGDHc activity was assayed at 37 ^{0}C in cell-free extracts from tellurite-exposed *E. coli* (0.5 µg/ml, 30 min). NAD^+ reduction was monitored at 340 nm for 1 min. The reaction mixture (1 ml) contained 25 mM Tris-HCl buffer, pH 7.0, 0.5 mM NAD^+, 10 mM KG, 20 mM $MgSO_4$, 1 mM CoASH and 4 mM TPP. Assays were started with the extract (100 µg protein) [16]. Blue native polyacrylamide gels were run for in-gel visualization of enzyme activity. Assays were started with the extract (300 µg protein) coupling NADH/NADPH formation to 0.3 mg/ml phenazine methosulfate and 0.5 mg/ml iodonitrotetrazolium as decribed [17].

Complex I and NADH oxidase activity was assessed at 37 ^{0}C in cell-free extracts from tellurite-exposed *E. coli* (0.5 µg/ml, 30 min). Blue native polyacrylamide gels were run for in-gel visualization of enzyme activity and then incubated with 25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ buffer for 15 min. Assays were started with the extract (100 µg protein), after adding 5 mM NADH and 5 mM KCN, 0.4 mg/ml iodonitrotetrazolium and 0.2 mg/ml dichlorophenol indophenol were used to reveal the activity [17].

Data analysis

In general, results were expressed as the mean ± the standard deviation. Differences between experimental groups were analyzed using one-way ANOVA. P values less than 0.05 were considered statistically significant.

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Results and Discussion

E. coli exposure to tellurite results in decreased KGDHc activity and unchanged complex I and NADH oxidase activities

To assess the effect of tellurite on KGDHc activity, extracts from tellurite-exposed cells were used. As determined by a spectrophotometric assay, enzymatic activity decreased ~30% when wild type *E. coli* was exposed to the toxicant for 30 min (Fig. S1A). KGDHc activity was also assessed *in situ*, after fractionating crude extracts from cells exposed or not to tellurite by non-denaturing polyacrylamide gel electrophoresis. KGDHc activity was practically undetected even after 24 h exposure (Fig. 1A). KGDHc activity abolition could be the consequence of a direct effect of tellurite on enzyme activity/structure or indirect, at the transcriptional level thus affecting mRNA synthesis for one (or more) KGDH components (see below).

On the other hand, it has been proposed that KG decarboxylation -with the concomitant NADH formation- would increase the oxidative status of the cell [18]. KGDHc inhibition/inactivation would help to alleviate the effects of tellurite-mediated oxidative damage. In this context, there are other examples regarding the role that metabolic enzymes could play in controlling oxidative damage caused by certain elicitors as aluminium [19] or tellurite [8, 9].

Given that limiting NADH production is crucial under oxidative stress conditions and since the activity of NADH-using enzymes usually decreases in

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these circumstances [20], the activity of complex I and NADH oxidase was assessed. No significant changes in these activities were observed in tellurite-exposed cells when compared with untreated controls (Fig. 1B), evidencing the importance of decreasing NADH levels through KGDHc inhibition to alleviate tellurite-mediated oxidative stress.

KG accumulates in tellurite-exposed *E. coli* cells

Since KGDHc activity decreases in tellurite-exposed *E. coli*, KG content was assessed by high performance liquid chromatography (HPLC). Higher KG levels were found in tellurite-exposed wild type cells in regard to untreated controls (Fig. 2A). These results suggest that KG may accumulate because of the decrease of KGDHc activity. Results supporting this came from the observation that decreased ICDH and GDH KG-synthesizing activities were found in toxicant-exposed *E. coli* (unpublished observations). All of these findings could help the bacterium in facing oxidative stress.

Conversely to KG, succinate content decreased in tellurite-treated cells (Fig. 2B). Since KG decarboxylation occurs non enzymatically in the presence of H_2O_2 or superoxide to yield succinate and CO_2 [12], one would expect to some extent that the increased KG content observed in tellurite-exposed cells results in augmented succinate levels. Probably succinate amounts could be augmented only after enough KG is accumulated and thus succinate coming from KG

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227 decarboxylation would serve to keep the Krebs cycle at work under basal
228 conditions.
229
230 Transcriptional level of *sucA*, *sucB* and *lpdA* in tellurite-exposed *E. coli*
231
232 To assess if the observed decrease of KGDHc activity in tellurite-treated
233 cells was related to the amount of *sucA*, *sucB* or *lpdA* transcripts, their levels were
234 analyzed by qPCR. Significant changes were observed only for the *sucA* gene.
235 While *sucB* and *lpdA* transcription was not altered significantly, that of *sucA* was
236 decreased by ~50% (Fig. S1B). Although not totally conclusive, these results
237 possibly reveal that tellurite inhibits KGDHc activity by affecting the amount of the
238 E1 component transcript rather that exerting a direct effect on the $[(SucA)_{12}$-
239 $(SucB)_{24}$-$(Lpd)_2]$ multienzyme complex. Decreased *sucA* transcription would result
240 in less SucA to form further complexes and hence in decreased enzymatic activity,
241 which also would slow the functioning of the Krebs cycle. Decreased *E. coli sucA*
242 transcription has also been observed in *E. coli* cells exposed toTiO_2 [21].
243
244 Tellurite susceptibility of *E. coli* deficient in KG metabolism
245
246 A wild type *E. coli* as well as Δ*sucA*, Δ*sucB* and Δ*lpdA* strains was analyzed
247 to assess tellurite tolerance. All mutant derivatives showed increased tellurite
248 sensitivity in regard to the isogenic, parental strain (Table 2). Particularly
249 interesting was the Δ*lpdA* strain, which was eight-fold more sensitive to tellurite

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than wild type cells. It should be noted that tellurite concentrations used in these experiments could affect, in addition to the Krebs cycle, other metabolic pathways such as glycolisis [22], the pentose phosphate shunt and/or the electron transport chain (unpublished data), which would inhibit bacterial growth.

ROS content in tellurite-exposed *E. coli*

Total ROS as well as superoxide content was assessed in tellurite-exposed *E. coli*. Regarding the respective untreated controls, increased levels of superoxide and total ROS were observed in all tellurite-treated cells (Figs. 3 and 4). Increased levels of these species were more evident in the wild type strain. At least 20% less total ROS was observed in tellurite-treated mutant strains as compared to the wild type counterpart. This could be interpreted as the lack of KGDHc activity in mutant strains resulting in KG accumulation which could be used in ROS scavenging.

Finally, all tested strains exhibited high basal superoxide levels. This could be explained because KGDHc inhibition/inactivation would interrupt the functioning of the Krebs cycle, which in turn would result in a decreased cellular antioxidant pool. In addition, dihydroethidine becomes very toxic after being oxidized by superoxide because of its interaction with DNA [23]. Regarding the respective controls, fluorescence intensity increased 37, 28, 7 and 28% in tellurite-exposed wild type, Δ*sucA*, Δ*sucB* and Δ*lpdA*, respectively. These results support those obtained when analyzing total ROS content. To shed further light to the tellurite

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effect on global KG metabolism, experiments regarding KG biosynthesis upon tellurite exposure are currently being carried out in our laboratory.

Acknowledgements

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386 **Figure legends**

387

388 **Fig. 1**. *In situ* KGDHc, complex I and NADH oxidase activity. KGDHc (A) and
389 complex I and NADH oxidase activities (B) were assayed after fractionating
390 extracts from tellurite-treated cells by native gradient polyacrylamide gels as
391 described in Methods. Representative gels are shown.

392

393 **Fig. 2**. Metabolite assessment in tellurite-exposed wild type *E. coli*. KG (A) and
394 succinate (B) contents in wild type *E. coli* exposed to 0.5 µg/ml tellurite for 5, 15
395 and 30 min. were assessed by HPLC as described in Methods. The line represent
396 negative control for both compounds (no tellurite added). Numbers represent the
397 mean of 3 independent trials. ns, non significant.

398

399 **Fig. 3.** Total ROS levels in tellurite-exposed *E. coli*. The indicated *E. coli* strains
400 exposed or not to tellurite or tert-butyl hydroperoxide (TBH) were assessed for total
401 ROS content by flow cytometry using 2',7'-dihydrodichlorofluorescein diacetate as
402 described in Methods. Dot Plot representation, X axis represents fluorescence
403 intensity and Y axis forward scattering (FCS). A, wild type; B, Δ*sucA*; C, Δ*sucB*; D,
404 Δ*lpdA*.

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Fig. 4. Superoxide generation in tellurite-exposed *E. coli***.** The indicated *E. coli* strains exposed or not to tellurite were assessed for superoxide content by flow cytometry using dihydroethidine as described in Methods. Dot Plot representation, X axis represents fluorescence intensity and Y axis forward scattering (FCS). A, wild type; B, Δ*sodAB*; C, Δ*sucA*; D, Δ*sucB*; E, Δ*lpdA*.

Fig. S1. KGDHc activity and transcriptional level of KGDH-encoding genes in tellurite-exposed *E. coli*. A, spectrophotometric determination of KGDHc activity in extracts of wild type cells; B, Transcriptional level of KGDH-encoding genes in tellurite-exposed *E. coli*. Values above or below the line indicate increased or decreased transcription, respectively. Numbers represent the mean of 3 independent trials.

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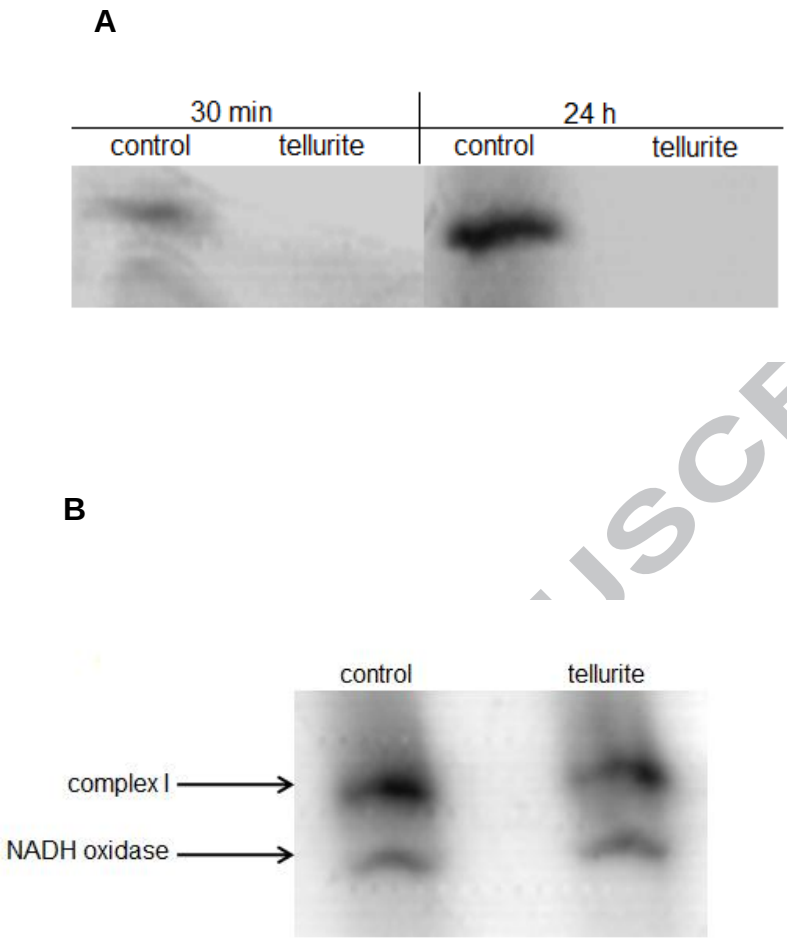

Fig. 1. Reinoso *et al.*

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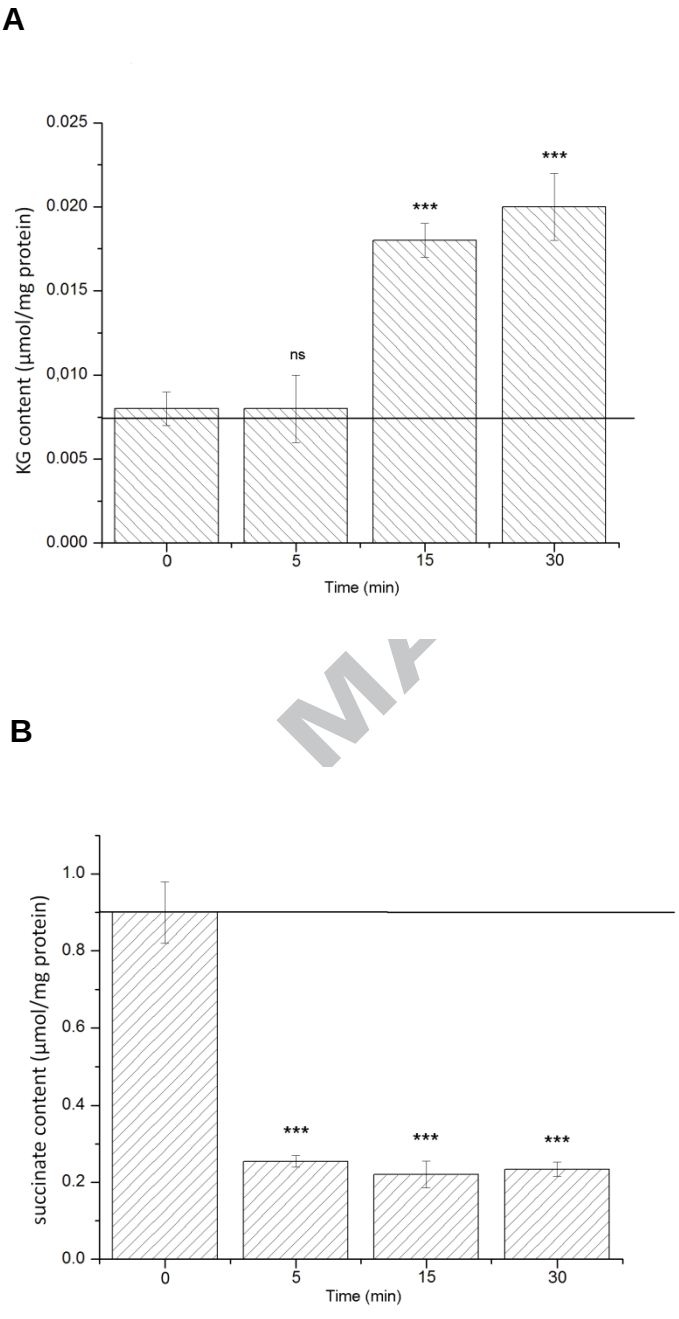

Fig. 2. Reinoso *et al.*

Figure 3

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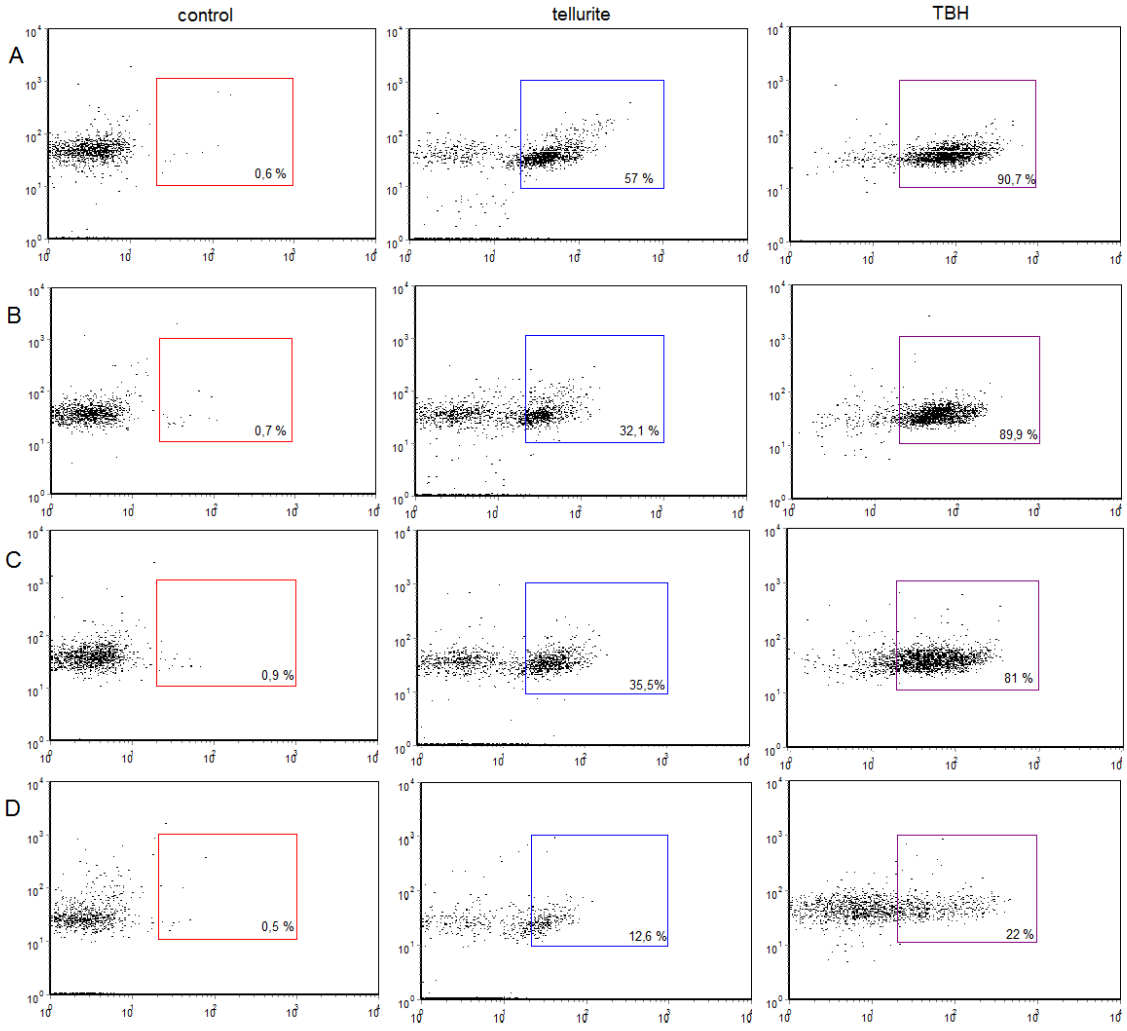

Fig. 3. Reinoso *et al.*

Figure 4

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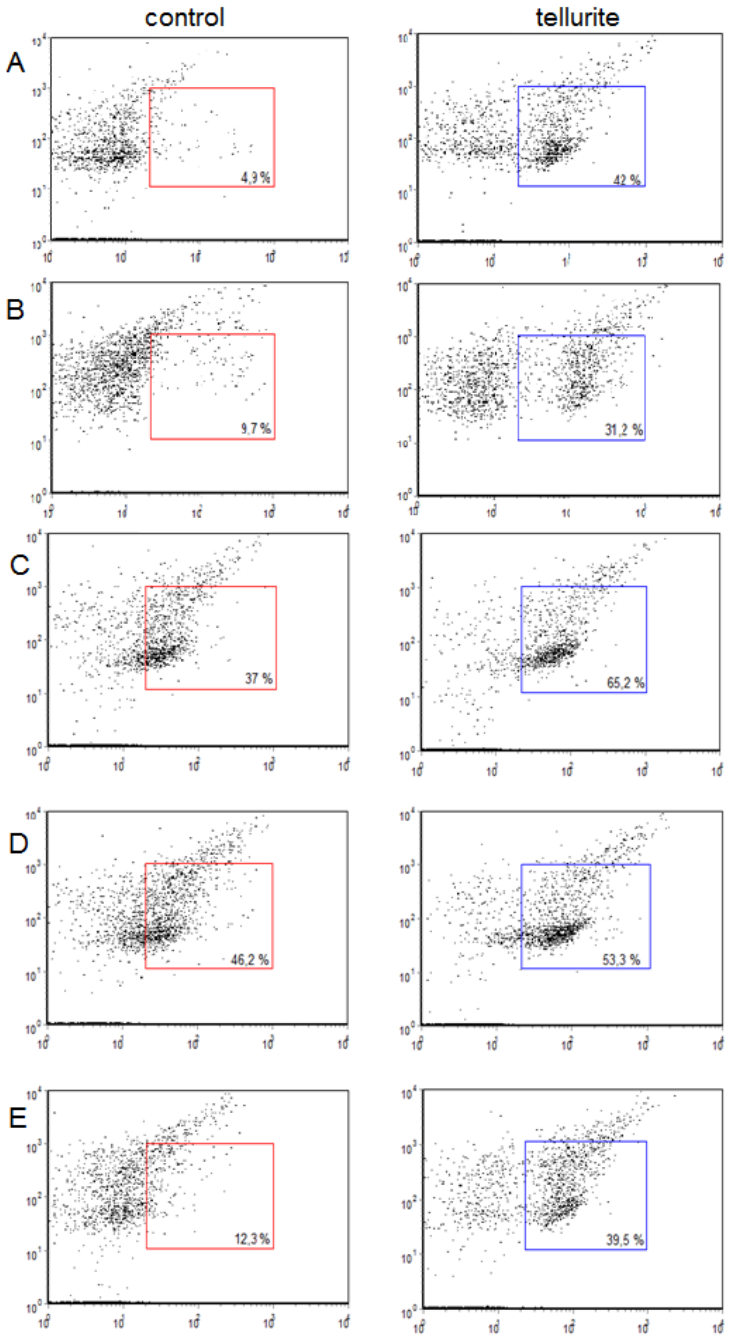

Fig. 4. Reinoso *et al.*

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Table 1. *E. coli* strains and primers used in this study

Strain	Relevant genotype	Source or reference
BW25113	Δ*(araD-araB)567*, Δ*lacZ4787*(::*rrnB*-3),	Baba et al. 2006
Δ*sucA*	BW25113 *sucA* (*icdA*::Kan^R)	Baba et al. 2006
Δ*sucB*	BW25113 *sucB* (sucB::Kan^R)	Baba et al. 2006
Δ*lpdA*	BW25113 *lpdA* (*lpdA*::Kan^R)	Baba et al. 2006

Primers	Forward (F) or Reverse (R), to amplify	5′-3′ Sequence
sucA F	F, *sucA*	ATGCAGAACAGCGCTTTGAA
sucA R	R, *sucA*	CGGAAATATTCACGCGTTTG
sucB F	F, *sucB*	CTGACCTGCCTGAATCCGTA
sucB R	R, *sucB*	ACCAAGGATCTGACGAGACG
lpdA F	F, *lpdA*	GTACTGAAATCAAAACTCAGGTCG
lpdA R	R, *lpdA*	CGCTTTGGCTTCTTCGATAA

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Table 2. Tellurite GIZ (cm^2) and MIC (µg/ml) for the indicated *E. coli* strains

Strains	GIZ	MIC
BW25113	7.1	0.8
Δ*sucA*	7.4	0.4
Δ*sucB*	7.8	0.4
Δ*lpdA*	9.9	0.1

GIZs and MICs were determined in LB medium as described in Methods. Numbers are the mean of 3 independent trials.

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1.- Tellurite-exposed *E. coli* exhibits decreased α-KG dehydrogenase activity

2.- Cells lacking α-KGDH genes are more sensitive to ROS than isogenic, wt *E. coli*

3.- KG accumulation may serve to face tellurite-mediated oxidative damage in *E. coli*

ACCEPTED MANUSCRIPT