Chemico-Biological Interactions 305 (2019) 195–202

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Evaluation of the promiscuous component of several bacterial export pumps TolC as a biomarker for toxic pollutants in feedstuffs

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ARTICLE INFO

Keywords:
Food safety
Biosensor
Chemoresistance
Escherichia coli
Export pump
Toxicity

ABSTRACT

A significant risk to the food chain is the presence of noxious pollutants in the feeds of animals whose products are used in human nutrition. Consequently, analytical methods and biosensors have been developed to detect these types of contaminates in feeds. Here we have evaluated whether the expression of TolC, a promiscuous component of several ATP-dependent efflux pumps in *E. coli*, up-regulated in response to chemical stress, could be a useful biomarker for this aim. Changes in TolC expression in response to toxic compounds, with different abilities to induce DNA damage, were determined using two *E. coli* strains with (DH5α) and without (BL21(DE3)) inactivating mutation in *RecA* gene. Deoxycholic acid and potassium dichromate up-regulated TolC in both strains. In contrast, cisplatin-induced TolC up-regulation was abolished in the absence of a functional RecA. When the effect of several insecticides, herbicides, antibiotics and common soil pollutants on TolC expression was analyzed, a relationship between toxicity and their ability to up-regulate TolC was observed. However, this was not a general event because the insecticide α-cipermetrin induced a reduction in cell viability, which was not accompanied by TolC up-regulation. In contrast, the soil pollutant benzene was able to stimulate TolC expression at non-toxic concentrations. When this test was used to analyze aqueous extracts from different feedstuffs, up-regulation of TolC was found in the absence of cell toxicity and was even accompanied by enhanced cell viability. In conclusion, TolC expression is partly dependent on the integrity of the RecA/LexaA system. Although toxic compounds up-regulate TolC in a dose-dependent manner, this response is also activated by non-toxic agents. Thus, owing to its poor specificity regardless of its sensitivity, the use of TolC up-regulation in *E. coli* to detect the presence of toxic pollutants in conventional and unconventional sources of nutrients for ruminant feeding requires supplementary biomarkers.

1. Introduction

High levels of environmental pollution together with the increasing use of fertilizers, pesticides, plasticizers, hydrocarbons and other chemicals in the agricultural production of animal feeds as well as the addition of unconventional raw materials may increase the risk of unwanted incorporation of noxious substances, particularly persistent organic pollutants, to the human food chain [1]. This is an important and general concern that has led numerous administrations to invest in the prevention of this serious danger. To protect consumers from exposure to food containing potentially harmful residues, specific European legislation (European Regulation No 470/2009) has established what is known as Maximum Residue Limits (MRLs). This fix the amounts of pesticides, veterinary medicines, mainly antibiotics, and environmental contaminants that can be considered as acceptable since these compounds pose a serious risk to the health of animals and humans health through the promotion of antibiotic resistance.

Different methods have been explored that include physical, chemical and biological approaches. But unfortunately, these methods are not able to sufficiently and safely clean the environment from the continuous production of toxic pollutants caused by anthropogenic activities. Novel methods based on bacterial enzymes are easy, quick,

Abbreviations: ABC proteins, ATP-binding cassette proteins; CDDP, cis-diamminedichloroplatinium (II); DCA, deoxycholic acid; MFS, major facilitator transporters; KDC, potassium dichromate

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https://doi.org/10.1016/j.cbi.2019.03.028
Received 27 September 2018; Received in revised form 31 January 2019; Accepted 26 March 2019
Available online 30 March 2019
0009-2797/

eco-friendly and socially acceptable alternatives for the elimination of recalcitrant xenobiotic toxic compounds that may be incorporated into agricultural products [2,3].

Additionally, there is a growing need for the development of safety controls for the sensitive detection of toxic compounds, which would prevent the carryover of harmful pollutants into the food chain. The first step to address this problem would be to design screening methods capable of detecting the presence of noxious residues in animal feeds. The ideal procedure must be simple, fast, sensitive, inexpensive and useful for a wide range of molecules. With this aim in mind, many biosensors have been developed in the field of alimentary toxicology, some of which are based on the use of living organisms, including bacteria. The purpose of fusing microorganisms is to force them to trigger measurable reactions in response to the presence of toxic chemicals in samples being analyzed. The bacterial stress response is part of the SOS machinery of these cells and is comprised of a variety of defense strategies, such as changes in membrane composition and up-regulation of genes involved in antioxidant reactions, DNA repair, xenobiotic metabolism and export of toxic compounds [4,5].

The efflux of noxious molecules in gram-negative bacteria involves highly conserved mechanisms that require the combined action of outer efflux proteins (OEP), transport proteins located at the inner membrane, and periplasmic proteins called membrane fusion proteins (MFP) that mediate the association between the transporters and OEPs. In *Escherichia coli*, at least 37 putative inner multidrug transporters (MDTs) have been identified, which belong to five different families: major facilitator (MFS), small multidrug resistance (SMR), resistance nodulation cell division (RND), ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) [6]. In addition, many ABC and MFS proteins require TolC to form trans-periplasmic complexes that efficiently export small molecules and toxins across the outer membrane of gram-negative bacteria [7,8] (Fig. 1A). TolC proteins constitute a large family of members expressed in many gram-negative bacteria. These proteins are able to interact not only with different inner membrane transporters [8] but also with the majority of MFPs expressed in these bacteria [9]. Depending on the MDT with which TolC interacts, these proteins can transport a broad range of different substrates as part of a multidrug resistance (MDR) mechanism, playing a critical role in conferring antibiotic resistance. Among these substrates are proteins that are toxic to its host, such as α-hemolysin, colicin V or microcins [10–13], metabolites such as cAMP [14], porphyrins [15] and cysteine [16], and a large variety of xenobiotic substances such as detergents, dyes, organic solvents and multiple antibacterial agents [17].

Even though the *TolC* gene is constitutively transcribed in *E. coli*, its expression, which is independent from the inner membrane counterparts, is further upregulated in response to different environmental signals, including low Mg^{2+} concentrations [18], the presence of salicylate, paraquat, bile salts, decanoate [19], indole, ethanol, EDTA, flavonoids and sodium tungstate [20,21], as well as environmental acidification [22,23]. The exact mechanism controlling TolC expression is still poorly understood; however, there is evidence that many different signals modulate the activity of multiple transcriptional regulatory elements. It is known that four different promoters control TolC levels according to specific conditions [24], allowing cell to adapt to different environments. Furthermore, small regulatory RNAs (sRNAs) are known to be major posttranscriptional regulators that can enhance or repress translation by binding to the 5′-untranslated region (UTR) of mRNA targets. SdsR (also known as RyeB) is a well-conserved non-coding RNA that is abundant in stationary-phase and has been found to repress TolC expression [25].

The aim of this study was to analyze in *E. coli* enhanced TolC expression and its potential usefulness as a biomarker for the detection of pollutants in different conventional and unconventional materials used in ruminant feeds.

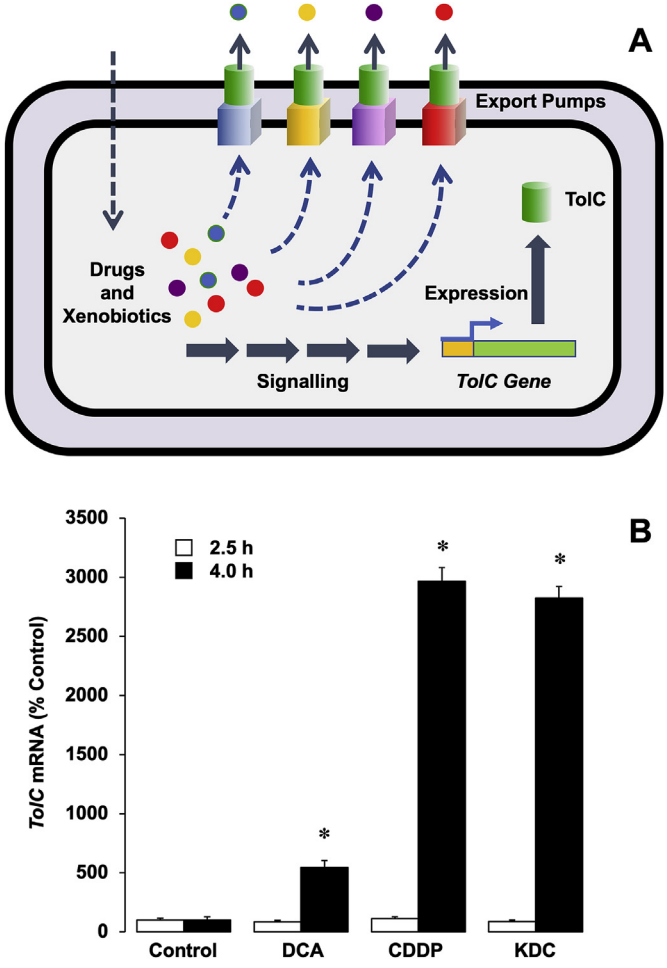

Fig. 1. (A) Scheme depicting the role of TolC as a promiscuous component of drugs and xenobiotic export pumps in bacteria and its response to the toxic challenge. (B) Time course of *TolC* expression by BL21(DE3) *E. coli* in response to deoxycholic acid (DCA, 2 mM), cisplatin (CDDP, 50 μM) or potassium dichromate (KDC, 400 μM). Values are means ± SEM (n = 4). *, $p < 0.05$ as compared to Control by the Student *t*-test.

2. Materials and methods

2.1. Chemicals

Ampicillin, chloramphenicol, salicylic acid, cis-diamminedichloroplatinium (II) (CDDP), sodium deoxycholate (DCA), diuron, paraquat dichloride hydrate, diazinon, α-cypermethrin, bis (2-ethylhexyl) phthalate (BEHP) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Quimica (Madrid, Spain). Imidacloprid was supplied by Sigma-Aldrich (St. Louis, MO). Tryptone, yeast extract and agar were obtained from Pronadisa (Laboratorios Conda, Madrid, Spain) potassium dichromate (KDC) was from Panreac Quimica (Barcelona, Spain) and benzene was from Applichem (VWR, Barcelona, Spain).

2.2. Aqueous extracts

Several conventional and unconventional feeds for ruminants were analyzed (Table 1). These feeds included ashes, obtained from the combustion of either recycled/residual wood or dry olive cake, and conventional and unconventional materials used at that aim. They were provided as dry powder by the “Estación Experimental del Zaidín” (EEZ) (Granada, Spain). Immediately prior to use, aqueous extracts (0.5 g/ml) of the samples were prepared in ultrapure water and filtered

Table 1
Materials used in the present study to obtain aqueous extracts.

Type	Code	Origin	Characteristics
Ashes	AS1	Ence, Huelva (Spain)	Obtained from the combustion of recycled/residual wood
	AS2	Baena, Cordoba (Spain)	Obtained from the combustion of wet olive cake
	AS3	La Loma, Jaen (Spain)	Obtained from the combustion of dry olive cake
	AS4	Tradema, Jaen (Spain)	Obtained from the combustion of recycled/residual wood
Dry feeds	DV1	EEZ, Granada (Spain)	Pellets including dry wastes of tomato fruits
	DV2	EEZ, Granada (Spain)	Alfalfa hay harvested in 2014
	DV3	EEZ, Granada (Spain)	Wastes of cucumber fruits
	DV4	EEZ, Granada (Spain)	Multinutrient block including mango pulp and peel
	DV5	EEZ, Granada (Spain)	Lyophilized wastes of cucumber fruits
	DV6	EEZ, Granada (Spain)	Alfalfa hay harvested in 2016
	DV7	EEZ, Granada (Spain)	Concentrate based on a seeds mixture
	DV8	EEZ, Granada (Spain)	Lyophilized wastes of tomato fruits
	DV9	EEZ, Granada (Spain)	Lyophilized mixture of avocado pulp and peel
	DV10	Granada cost (Spain)	Lyophilized wastes of tomato fruits
	DV11	Granada cost (Spain)	Lyophilized wastes of ecological tomato fruits
Algae	AP1	Bødo cost (Norway)	Multinutrient block including the seaweed *Porphyra sp.* collected in 2016
	AP2	Bødo cost (Norway)	Seaweed collected in 2016
	AP3	Bødo cost (Norway)	Multinutrient block based on conventional raw materials
	AP4	Bødo cost (Norway)	Multinutrient block including the seaweed *Saccharina latissima* collected in 2016
	AP5	Tjøtta (Norway)	Multinutrient block based on conventional raw materials collected in 2017
	AP6	Tjøtta (Norway)	Multinutrient block including the seaweed *Porphyra sp.* collected in 2017
	AP7	Tjøtta (Norway)	Multinutrient block including the seaweed *Saccharina latissima* collected in 2017
Diets	AD1	EEZ (CSIC)	Commercial concentrate
	AD2	Tjøtta (Norway)	Diet based on silage and oat
	AD3	Tjøtta (Norway)	Diet based on silage, oat and clover
	AD4	Tjøtta (Norway)	Diet based on silage, oat and soya
	AD5	Tjøtta (Norway)	Diet based on silage, oat and *Porphyra sp.*

EEZ, Estación Experimental del Zaidín, Granada, Spain.

(0.22 μm-pore size filter) as previously reported [26]. The pH of bacterial cultures was measured after adding the aqueous extracts. It was not necessary to adjust the pH in any case.

2.3. Bacterial strains, growth conditions and toxicity tests

E. coli DH5α (F– Φ80lacZΔM15, Δ(lacZYA-argF), U169 RecA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1) was obtained from Invitrogen (Life Technologies, Madrid, Spain). The strain of *E. coli* BL21(DE3) *(F– ompT hsdSB(rB–, mB–) gal dcm (DE3))* was donated by the Department of Microbiology and Genetics, University of Salamanca (Spain). The *E. coli* cells were grown on Luria-Bertani (LB) agar plates and used to inoculate LB liquid cultures, which were incubated with shaking (220 rpm) at 37 °C until reaching the stationary phase. For the assays, overnight cultures were diluted 1:1,000 in fresh LB. Then, 6 ml were added to ventilated test tubes containing 20 μl of the aqueous extracts or toxic compounds at different concentrations and incubated for 4 h at 37 °C with shaking. Lastly, aliquots from the bacterial culture were collected and bacterial growth was analyzed by measuring the optical density (OD_{600}), or gene expression by RT-qPCR.

2.4. TolC gene expression

Total RNA from BL21(DE3) and DH5α *E. coli* ($\approx 5 \times 10^8$ cells) was stabilized using the RNA-protect Bacteria Reagent (Qiagen, Izasa Scientific, Barcelona, Spain). RNA was isolated after lysozyme-proteinase K digestion using RNeasy Mini Kit (Qiagen) and On-Column DNase I digestion kit (Qiagen). RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain) and RNA quality was evaluated on a denaturing agarose gel. Reverse transcription (RT) was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). RT reactions without retrotranscriptase were used as the controls to confirm the lack of contaminating DNA in the RNA samples. The cDNA obtained was used for quantitative PCR (qPCR) in an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using SYBR Green I (Applied Biosystems), as previously described [27]. The oligonucleotide sequences of the primers and the thermal cycling conditions used for measuring the abundance of *TolC* mRNA and 16S rRNA, used as the normalizer, are shown in the Supplementary Table 1.

2.5. Statistical analyses

Data are shown as mean ± SEM. After ANOVA, the statistical significance of the differences ($p < 0.05$) among groups was determined using the unpaired *t*-test.

3. Results

To optimize the experimental conditions of the assay, a pilot study was carried out by exposing the bacteria to known toxic compounds, such as DCA, CDDP and KDC, for 2.5 and 4.0 h during the exponential-phase of cultures of the *E. coli* strain BL21(DE3). No change in *TolC* mRNA abundance was observed after 2.5 h of exposure (Fig. 1B). In contrast, a marked increase in TolC expression was observed 4.0 h after incubation with DCA, which was used as a positive control as it is known to be a strong TolC inducer [28]. In addition, a more marked response was obtained after incubation with CDDP and KDC (Fig. 1B).

Subsequently, the ability of TolC expression to respond to the toxic challenge induced by several compounds belonging to major groups of pollutants in agricultural activities, such as insecticides, herbicides, antibiotics and typical soil pollutants derived from industry, at non-toxic or moderately toxic concentrations, was checked (Fig. 2). The highest eincrease in TolC expression levels was detected in response to the antibacterial compounds ampicillin and chloramphenicol and the herbicide paraquat (Fig. 2A). In general, a direct relationship between toxicity and stimulation of TolC expression was found (Fig. 2B). However, this was not a general trend, because the insecticide α-cipermetrin induced a reduction in cell viability, which was not accompanied by TolC up-regulation. In contrast, benzene, classified here as a soil pollutant, was able to stimulate TolC expression at concentrations that were unable to induce toxic effects. In general, a dose-dependent response was observed for most chemicals assayed (Fig. 2A).

The effect of a set of aqueous extracts, obtained from samples whose

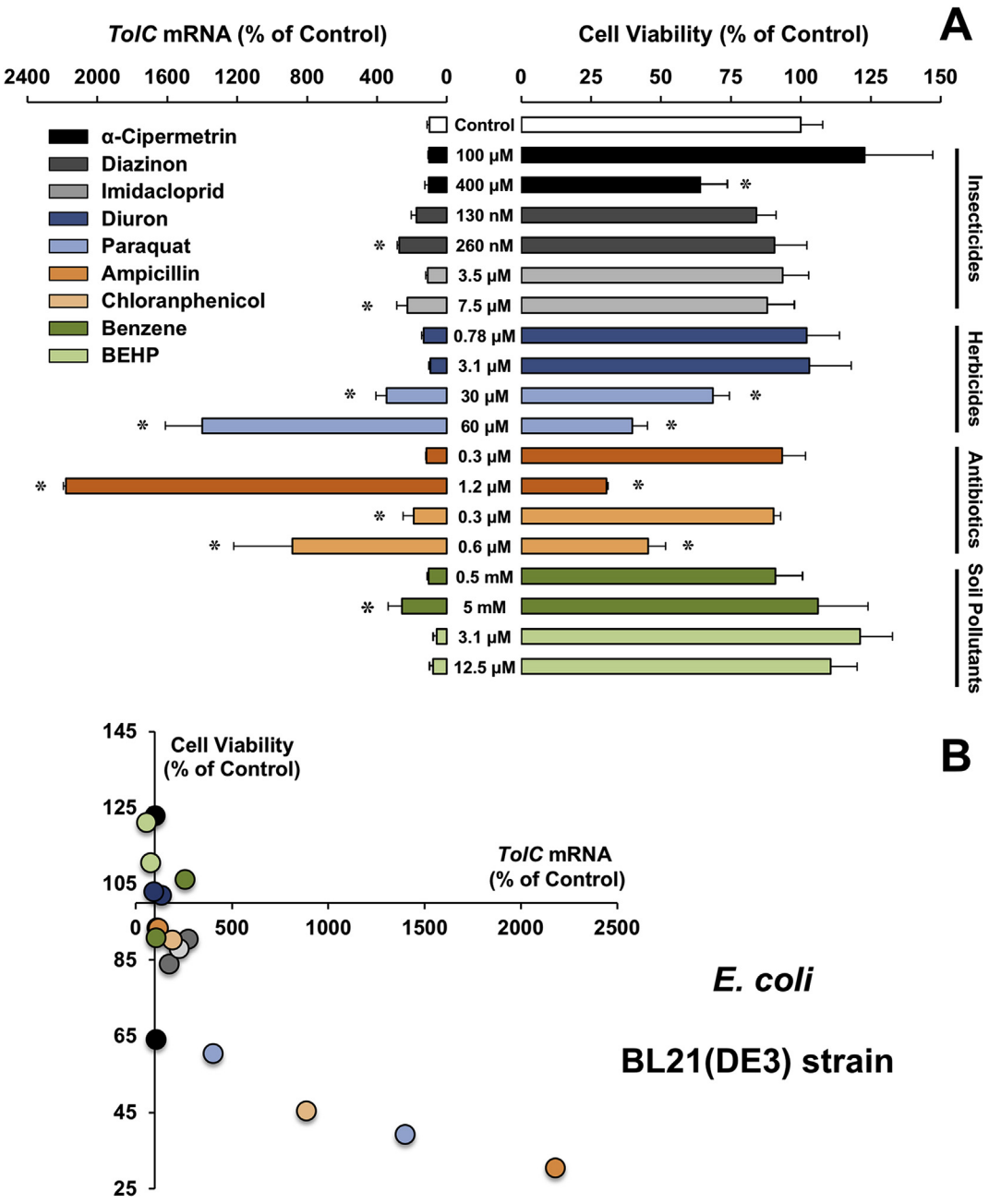

Fig. 2. (A) Effect of different types of agricultural soil pollutants on *TolC* expression and cell toxicity in *E. coli* strain BL21 (DE3). Changes in mRNA abundance and culture growth were determined by RT-qPCR and by measuring the optical density at 600 nm, respectively, in samples collected after incubation with non-toxic and sub-toxic doses of the indicated compounds at 37 °C with gentle shacking for 4 h. (B) Relationship between *TolC* expression and cell viability. Values, represented as a percentage as compared to the control, are expressed as mean ± SEM from 9 incubations carried out in three separate experiments. *, $p < 0.05$ as compared to Control by the Student *t*-test.

characteristics are shown in Table 1, on TolC expression was then examined. After carrying out concentration dependent measurement of bacterial cell viability (data not shown), we have selected non-toxic concentrations for each extract. The presence of some of these extracts in the culture medium markedly stimulated TolC expression (Fig. 3A) without reducing cell viability (Fig. 3B). Some of the ash samples (AS) used here are known to contain a certain degree of pollutants, such as lead (Pb) (AS3 and AS4) and chromium (Cr) (AS1, AS3 and AS4) [26]. Interestingly, the sample AS4, with the highest content in heavy metals both Pb (432 mg/kg ashes) and Cr (323 mg/kg ashes) [26], was able to stimulate TolC expression the most, even at non-toxic concentrations (Fig. 3A), whereas AS1, with negligible Pb concentration and lower levels of Cr [26] was not able to up-regulate TolC.

The rest of the samples assayed were comprised of conventional and unconventional materials used for feeding ruminants. Consequently, it was assumed that the samples could contain amounts of heavy metals below the acceptable MRL for this particular use. It is worth noting that the extract able to induce one of the strongest responses, with respect to TolC up-regulation, was derived from tomatoes grown using organic farming (Fig. 3A). It is also relevant to note that several aqueous extracts were able to induce TolC expression as well as stimulate cell viability (Fig. 3B).

To elucidate whether TolC up-regulation was dependent on a RecA-mediated response to DNA damage we compared the responses of two strains of *E. coli* with and without an inactivating mutation in the *RecA* gene, DH5α and BL21(DE3), respectively, to compounds with different

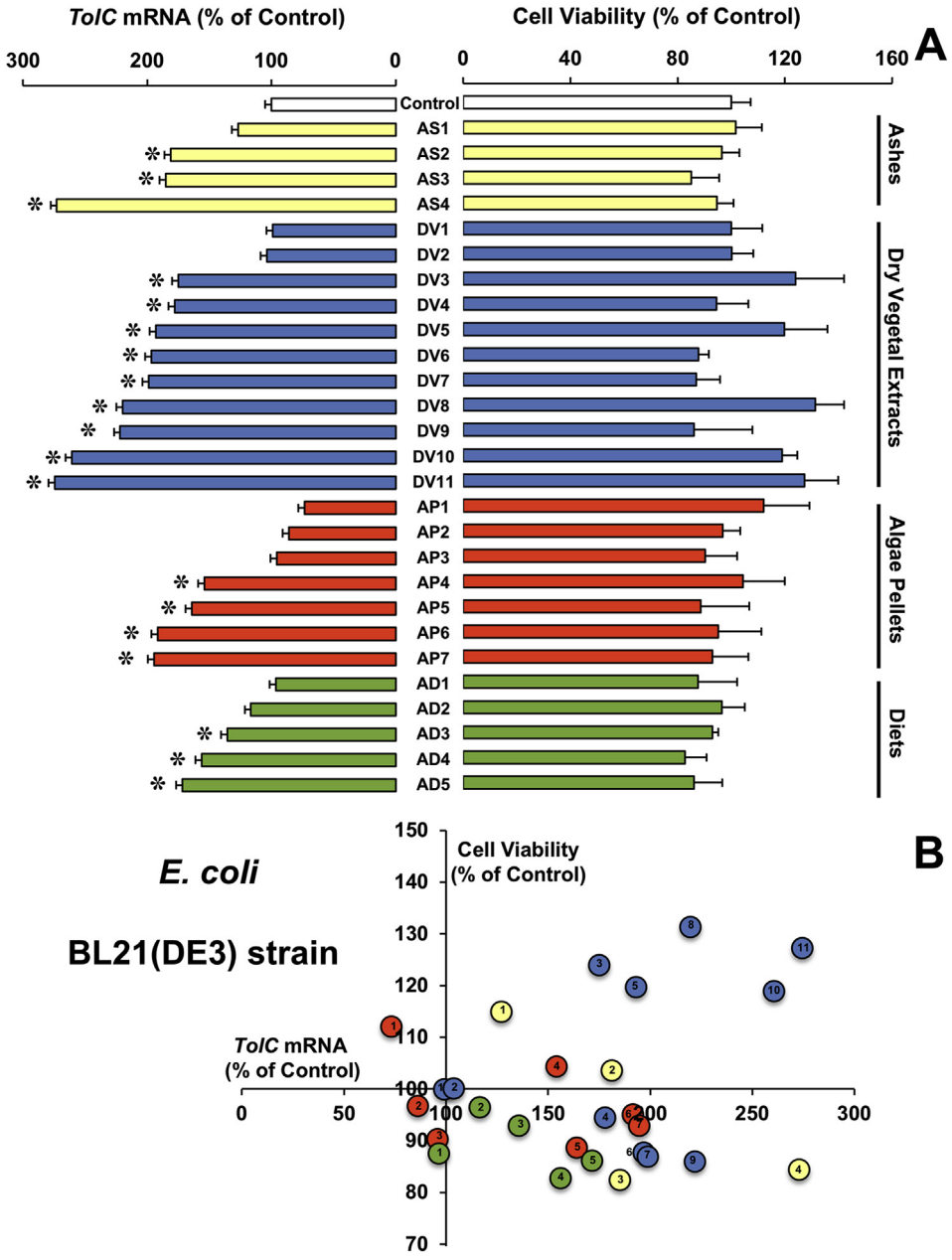

Fig. 3. (A) Effect of the aqueous extract obtained from ashes (AS), dry plant extracts (DV), algae pellets (AP) and animal feed (D) on *TolC* expression and cell toxicity in *E. coli* strain BL21 (DE3). Changes in mRNA abundance and culture growth were determined by RT-qPCR and by measuring the optical density at 600 nm, respectively, in samples collected after incubation with non-toxic and sub-toxic doses of the indicated compounds at 37 °C with gentle shacking for 4 h. (B) Relationship between TolC expression and cell viability. Values, represented as a percentage oas compared to the Control (water), are expressed as mean ± SEM from 9 incubations carried out in three separate experiments. *, $p < 0.05$ as compared to Control by the Student *t*-test.

genotoxic abilities (Fig. 4). In situations where cell viability was similar, the response was generally weaker in the DH5α cells, which were more resistant than the BL21(DE3) cells to salicylic acid and DCA. The response was much weaker for KDC in DH5α cells, whereas CDDP was unable to induce TolC up-regulation in these cells. Interestingly, the more chemoresistant strain had approximately five times higher TolC expression (Fig. 5), which may account for an enhanced ability to prevent intracellular accumulation of noxious compounds.

4. Discussion

Most of the studies devoted to the detection of environmental toxicity affecting the food chain have searched for biomarkers in laboratory animals exposed to different pollutants, such as pesticides, in their food. These included, for instance, oxidative stress biomarkers (lipid peroxidation and antioxidant enzymes in particular) in various tissues and cell compartments of mice and rats [29]. Other studies have focused their attention on the research concerning the effects of toxic pollutants, such as metal ions (particularly iron, copper, chromium, mercury, arsenic, nickel, etc.), pesticides (insecticides, herbicides, and fungicides) on aquatic organisms that may be useful as sensitive bioindicator of environmental pollution that can enter the food chain [30]. In homology to multidrug resistance (MDR), the concept of multixenobiotic resistance (MXR) refers to the response of microorganisms to the challenge of a toxic environment by expressing bacterial stress response genes. Several groups [31–34], including our laboratory [26] have previously explored the possibility of taking advantage MXR for developing novel biomarkers and biosensors.

Many bacterial species are able to tolerate antibiotics and other toxic compounds due, in part, to the activity of ATP-dependent transporters. In Gram-negative bacteria, such as *E. coli*, several of these efflux systems are formed by multicomponent structures that span both inner and outer membranes and are driven energetically by a primary or secondary transporter component. A model system for such pumps is the acridine resistance complex AcrAB-TolC, in which the outer-membrane channel is formed by the totally protein TolC [35] (Fig. 1A). Because efflux pumps are able to carry out vectorial transport that subsequently confers resistance to a large list of compounds with little

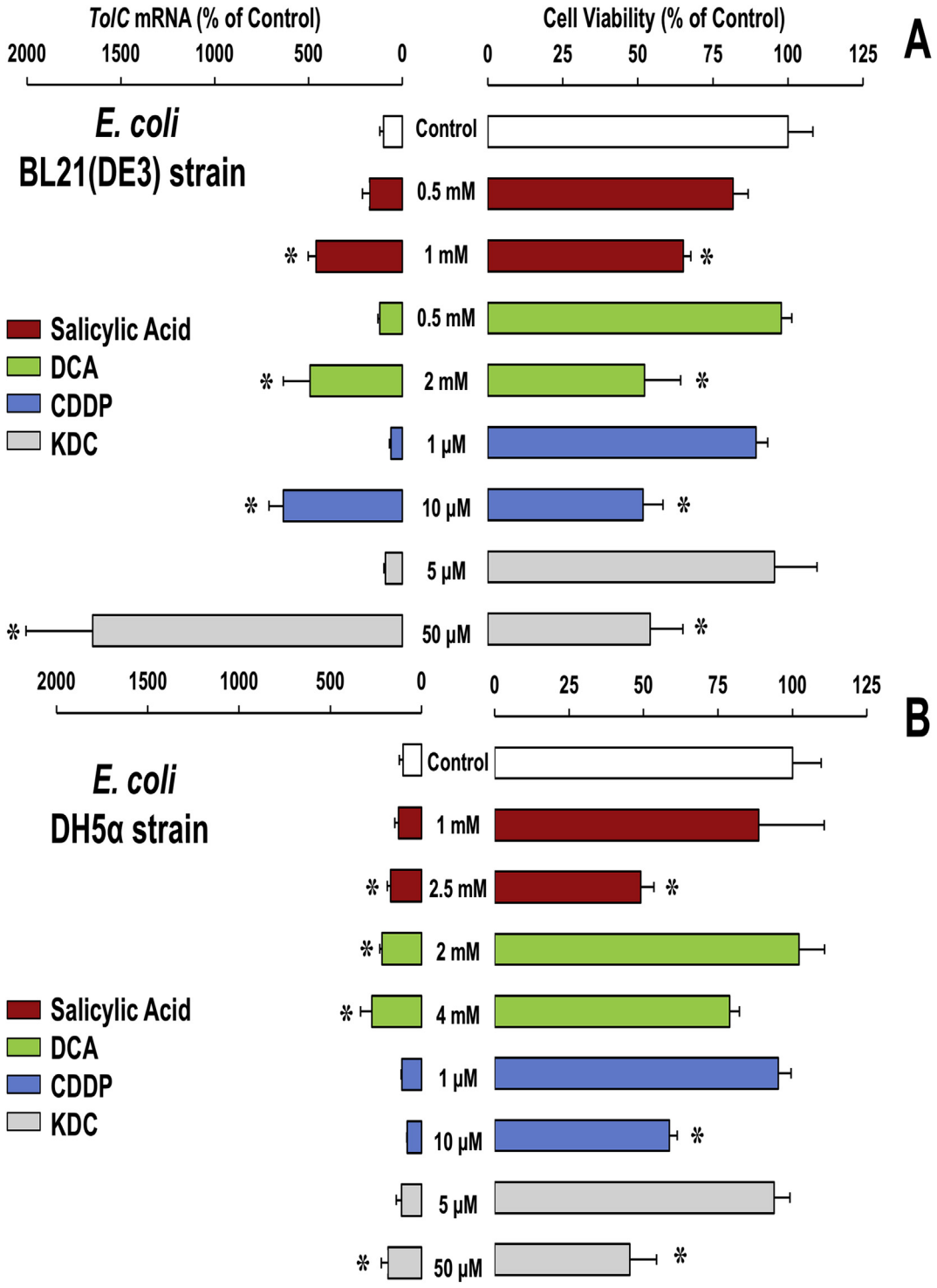

Fig. 4. Effect of chemicals with different genotoxic activity on *TolC* expression and cell toxicity in BL21(DE3) (A) and DH5α (B) strains of *E. coli*. Changes in mRNA abundance and culture growth were determined by RT-qPCR and by measuring the optical density at 600 nm, respectively, in samples collected after incubation with non-toxic and sub-toxic doses of the indicated compounds at 37 °C with gentle shacking for 4 h. Concentrations of deoxycholic acid (DCA) and cisplatin (CDDP) were adapted to the different levels of sensitivity of both strains to these compounds. Values, represented as a percentage as compared to the control, are expressed as mean ± SEM from 9 incubations carried out in three separate experiments. *, $p < 0.05$ as compared to Control by the Student *t*-test. KDC, potassium dichromate.

chemical similarity, it is reasonable to propose that the regulation of TolC expression in response to chemical stress could be used as a sensitive biomarker to detect the presence of toxic pollutants in early elements of the food chain, for instance in conventional and unconventional materials used for ruminant feeds. The present study has partly confirmed the initial hypothesis. Indeed, TolC expression was up-regulated by toxic compounds and extracts containing heavy metals but this response was also activated by non-polluted samples that presumably contain mixtures of compounds to bacteria. This does not mean, however, that these compounds are toxic, since some of the extracts not only triggered TolC up-regulation but also enhanced cell viability.

The results shown here indicate that most toxic compounds are able to up-regulate TolC, a response that becomes stronger as the toxic effect

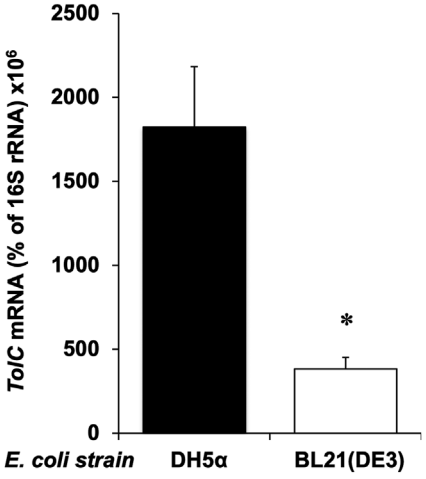

Fig. 5. Basal expression of *TolC* in BL21(DE3) and DH5α strains of *E. coli*. Abundance of *TolC* mRNA was determined by RT-qPCR and normalized by the amount of 16S rRNA. Values are mean ± SEM from 5 to 7 cultures, respectively. *, $p < 0.05$ as compared to *TolC* expression in *E. coli* DH5α strain by Student *t*-test.

challenges cell viability. This mechanism seems to be complex, since in the case of some toxic agents TolC up-regulation was observed in the absence of reduced cell viability. This has also been the case for many feeds studied that are able to enhance TolC at non-toxic concentrations. Some algae pellets and other raw materials induce TolC expression 2-fold, whereas for some ashes and feed extracts this increase is 3-fold. The complexity of the composition of the aqueous extracts obtained from these sources presumably accounts for the activation of more than one mechanism involved in the regulation of *TolC* gene.

The underlying pathway is easier to analyze in the case of single compounds. In a previous study [26], using the promoter of *E. coli* gene RecA linked to the ORF of firefly luciferase (RecApr–Luc2) we have developed a microbial biosensor to measure genotoxicity risks resulting from heavy metal pollutants in ashes that may be used as a mineral supplement in the elaboration of livestock feeds. The biosensor was based on the ability of the bacterial DNA repair system to be activated in response to genotoxic agents, which results in the expression of SOS genes that form part of a regulon [36]. In this system the RecA protein plays a key role as an inducer of genes involved in the SOS response [37]. In response to DNA damage, RecA binds to single-stranded DNA (ssDNA) to form a nucleoprotein filament [38]. Once bound to DNA, the co-protease activity of RecA is activated and then triggers the self-cleaving activity of the repressor LexA [39]. When this protein is cleaved, it dissociates from DNA and allows the transcription of the SOS genes [36].

The results obtained in the present study revealed that TolC up-regulation in response to genotoxic compounds, such as CDDP, is fully dependent on the integrity of the RecA/LexaA system. Thus, the DH5α strain characterized by the presence of inactivating mutations in the *RecA* gene was unable to up-regulate TolC in response to CDDP, whereas TolC expression was enhanced in BL21(DE3) cells, expressing functional RecA when incubated in the resence of CDDP. In contrast, the response against other agents, such as salicylic acid, DCA and KDC, which may induce DNA damage, is only partly dependent on functional RecA, because in its absence a reduced but not abolished TolC up-regulation was observed.

5. Conclusion

The present results indicate that TolC expression is only partly dependent on the integrity of the RecA/LexaA system. Moreover, although toxic compounds up-regulate TolC in a dose-dependent manner, this response is also activated by non-toxic agents. Thus, owing to its poor specificity in spite of its sensitivity, the use of TolC up-regulation in *E. coli* to detect the presence of toxic pollutants in conventional and unconventional sources of nutrients for ruminant feeding require supplementary biomarkers to discriminate false from true positive results.

Conflicts of interest

None.

Financial support

This study was supported by the Carlos III Institute of Health, Spain (Grants PI15/00179 and PI16/00598, co-financed by European Regional Development Fund -ERDF-); Ministry of Science and Innovation, Spain (SAF2013-40620-R and SAF2016-75197-R); “Asociación Española Contra el Cancer”, Spain (AECC-2017); “Junta de Castilla y León”, Spain (SA015U13 and SA063P17); “Fundación Samuel Solórzano Barruso, Spain (FS/08–2017 and FS/13–2017), Spain; “Fundación Mutua Madrileña”, Spain (Call 2015) and the Program of Excellence by the Junta de Andalucía, Spain (Project P12-AGR-587). Laura Pérez-Silva has been funded by the “Instituto de Salud Carlos III” and the “Fondo Social Europeo”, Spain (FI17/00149).

Acknowledgments

The authors thank J. Fernandez Yepes for the technical assistance and E. Keck for editing the English.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2019.03.028

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cbi.2019.03.028.

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