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Multidrug resistance pump AcrAB-TolC is required for high-level, Tet(A)-mediated tetracycline resistance in *Escherichia coli*

Ricardo E. de Cristóbal, Paula A. Vincent and Raúl A. Salomón*

Departamento de Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán) and Instituto de Química Biológica 'Dr Bernabé Bloj', Chacabuco 461, 4000 San Miguel de Tucumán, Tucumán, Argentina

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***Objectives*: Starting from the observation that *Escherichia coli tolC* mutations severely reduced the high-level resistance to tetracycline afforded by Tn*10*- and plasmid-encoded Tet(A) pumps, we studied the mechanism of this susceptibility.**

***Methods*: The MIC of tetracycline for MC4100 *tolC*::Tn*10* and several *tolC* mutants carrying the Tn*10* in other sites on the chromosome (*thr*::Tn*10*) was determined. The effect of a *tolC* mutation on the level of expression of Tn*10 tet*(A) was examined by using a *tet*(A)::*lacZ* gene fusion. Influence of *tolC* mutations on tetracycline efflux and accumulation was quantified by spectrofluorometric assays. The contribution of the AcrAB multidrug efflux system to high-level tetracycline resistance was measured in a Tn*10*-carrying *acrAB* null mutant strain.**

***Results*: Tn*10*- and plasmid-encoded Tet(A) conferred 5- to 6-fold lower levels of tetracycline resistance in *tolC* mutants, as compared with control strain *tolC*$^+$. Spectrofluorometric analyses showed that this resulted from a decrease in drug efflux in *tolC* mutants. Chlortetracycline resistance was also compromised by loss of TolC. Mutational loss of the AcrAB multidrug efflux transporter had the same effect as *tolC* mutations on tetracycline resistance. This indicated that *tolC* mutations act through inactivation of the AcrAB system.**

***Conclusions*: Our results are compatible with the hypothesis that the AcrAB pump is an important component in the development of high levels of resistance to tetracycline in *E. coli*, perhaps by working in combination with Tet(A).**

Keywords: *tolC* mutation, Tet(A)-AcrAB interplay, tetracycline susceptibility, *E. coli*

Introduction

Bacterial resistance to many classes of antibiotics is provided mainly by membrane transporter proteins called drug efflux pumps.[1] These pumps may occur as either single-component or multi-component systems. In Gram-negative bacteria, single-component efflux pumps expel their substrates into the periplasmic space. An example of such single-component efflux pumps is the transposon-encoded, tetracycline-specific Tet(A).[2] Multi-component efflux pumps (which are found exclusively in Gram-negative bacteria) traverse both inner and outer membranes. The major antibiotic efflux activity of this type in *Escherichia coli* is mediated by the tripartite multidrug resistance pump AcrAB-TolC.[3–5] This complex consists of the inner-membrane component AcrB, belonging to the resistance-nodulation-division (RND) family of proteins,[6] the outer membrane channel TolC and a periplasmic linker protein, AcrA, which is a member of the membrane fusion protein (MFP) superfamily.[7] The latter was thought to bring into contact the membrane-associated efflux components. However, Tamura *et al.*[8] have recently proposed that AcrB and TolC first directly dock with each other and then the complex is stabilized by AcrA. This structural organization allows extrusion of substrates from the cell into the external medium, bypassing the periplasm and the outer membrane.[9]

By using *tolC* mutants of strains carrying the transposon Tn*10* we demonstrate in this work that the outer membrane protein

*Correspondence address. Departamento de Bioquímica de la Nutrición, INSIBIO, Chacabuco 461, 4000 San Miguel de Tucumán, Argentina. Tel/Fax: +54-381-4248921; E-mail: salomon@fbqf.unt.edu.ar

TolC is required for high-level resistance to tetracycline (>40 mg/L) afforded by the Tn*10*- and plasmid-encoded Tet(A) pumps. Our results support the conclusion that *tolC* mutations act through the inactivation of the AcrAB efflux system in determining increased susceptibility of Tn*10*-carrying *E. coli* strains to tetracycline. We propose that high-level resistance conferred by Tet(A) results from its cooperation with AcrAB, which would capture tetracycline from the periplasm, where it has been accumulated by the action of Tet(A), and then would extrude the drug directly into the external medium.

Materials and methods

Strains, plasmids and media

Strains and plasmids used in this work are listed in Table 1. Growth medium was Luria– Bertani (LB) broth. Antibiotics were used at the following concentrations: kanamycin, 30 mg/L; ampicillin, 50 mg/L; and tetracycline, as indicated. The *tolC*::Tn*10*, *tolC*::Tn*5* and *acrAB* derivatives of MC4100 were constructed by phage P1 transduction,[10] using strains CAG12184, SC44 and AG100A as donors, respectively. The MC4100 TolC$^-$ and AcrAB$^-$ transductants had the expected deoxycholate-susceptible phenotype. Batch cultures were grown at 37°C with aeration by shaking.

Chemicals

Tetracycline was obtained from ICN Biomedicals, Inc. (Irvine, CA, USA) and chlortetracycline (7-chlorotetracycline) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Determination of the MIC of tetracycline

MICs of tetracycline were determined by a colony forming assay. LB plates were prepared with increasing concentrations of tetracycline (ranging from 10 to 200 mg/L, with 10 mg/L increments). They were inoculated (0.1 mL) with a 10^{-4} dilution of stationary-phase cultures grown in LB supplemented with 10 mg/L tetracycline, such that about 10^5 bacteria were spread per plate, and incubated at 37°C for 14–18 h. The MIC was the lowest concentration of tetracycline required to completely inhibit colony formation.

Table 1. *E. coli* strains and plasmids used in the study

Strain	Genotype	Source[a]
MC4100	*araD139* Δ(*argF-lac*)*205* λ$^-$ *flbB5301 ptsF25 relA1 rpsL150 deoC1*	CGSC
CAG12184	MG1655 *tolC210*::Tn*10*	C. Gross
SC44	C600 *tolC*::Tn*5*	C. Wandersman
PB3	*lacY1* or *lacZ4*? *gal-6*? *hisG1* Δ*tolC5 uxaC201 rpsL8* or *rpsl104* or *rpsL17 malT1mtlA2*?	CGSC
A586	*thr-1 leuB6 fhuA21 lacY1 glnV44*(*AS*) λ$^-$ *rfbD1 tolC3 thi-1 pro-42*	CGSC
AG100	*argE3 thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) *supE44*	H. Nikaido
AG100A	AG100 Δ*acrAB*::Tn*903*, Kan^r	H. Nikaido
Plasmid		
pAX629	pACYC184-based plasmid carrying *tolC*$^+$ gene cloned	C. Wandersman
pRKH40	ColE1-derived vector expressing a *tet*(A)::*lacZ* fusion under the control of repressor TetR	S. Levy

[a]CGSC, *E. coli* Genetic Stock Center.

Tetracycline efflux and accumulation assays

Tetracycline release and accumulation were measured using spectrofluorometry, as described previously.[11] For the efflux assay, bacteria (about 10^8 cells/mL) grown in LB were centrifuged and resuspended in 2 mL aliquots of Mg^{2+} buffer (50% methanol, 10 mM Tris–HCl, pH 8, 0.1 mM $MgCl_2$, 0.2% glucose). At time zero, tetracycline (100 mg/L) was added and the fluorescence (excitation at 400 nm and emission at 520 nm) recorded for at least 10 min with a spectrofluorometer (Gilson). Tetracycline accumulation was determined by using bacterial suspensions in Mg^{2+} buffer, prepared as described above. Tetracycline was added at 100 mg/L, and after 15 min of incubation bacterial suspensions were centrifuged, the pellets were resuspended in 2 mL of Mg^{2+} buffer and the released fluorescence (excitation at 400 nm and emission at 520 nm) was immediately recorded with a spectrofluorometer (Gilson). In other experiments, the absolute amount of tetracycline accumulated was determined as indicated by Ball *et al*.[12] LB overnight cultures of the strains to be tested were appropriately diluted with LB so as to get an optical density at 600 nm of 0.8. Samples (1 mL) were centrifuged, washed with 100 mM Tris/HCl buffer, pH 8, and resuspended in 1 mL of 10 mM Tris/HCl buffer, pH 8. Tetracycline was added at 100 mg/L, and the mixture was incubated for 15 min. Bacteria were harvested and the pellet was disrupted with 5 M HCl (1 mL), which, after boiling for 10 min, quantitatively converts tetracycline into anhydrotetracycline.[13] Cooled samples were centrifuged to remove cell debris. The absorbance at 440 nm of the anhydrotetracycline contained in the supernatants was measured. The amount of anhydrotetracycline contained in these samples was determined with a standard curve (0–100 mg tetracycline/L). The experiment was repeated six times, and the results were expressed in terms of μg of tetracycline/mg of cell protein. To correct for the amount of external tetracycline trapped within the bacterial pellet after centrifugation, one of the assays was performed at 0–4°C.[14] Low temperature inhibits diffusion[15] and active transport through lipid bilayers, thus preventing uptake of tetracycline through the inner membrane. Under these conditions, the amount of drug trapped in the pellet was 1.5 μg/mg of protein. This value was used for correction.

Analytical methods

Bacterial cell protein was estimated by the Lowry method.[16] Prior to assay, cells were heated at 90°C in 1 M NaOH for 10 min to obtain complete solubilization. β-Galactosidase activity was determined and expressed according to Miller.[10]

Results and discussion

High-level tetracycline resistance afforded by Tn10-encoded Tet(A) is affected by tolC mutations

This study arose from the observation that when *tolC*::Tn*10* strains are selected with tetracycline at a concentration conventionally used for this antibiotic (10 mg/L) they form colonies smaller than those growing in the absence of tetracycline. We thought it likely that even in the presence of Tn*10* the strains were being partially inhibited by the antibiotic. We determined

Table 2. Tetracycline susceptibilities of *tolC* mutants[a]

Strain	MIC (mg/L)
MC4100 *thr*::Tn*10*	200
MC4100 *tolC*::Tn*10*	40
MC4100 *tolC*::Tn*5* *thr*::Tn*10*	40
PB3 *thr*::Tn*10*	40
A586 *thr*::Tn*10*	40
MC4100 *tolC*::Tn*10* (pAX629)	200
MC4100 (pBR322)	180
MC4100 *tolC*::Tn*10* (pBR322)	30
MC4100 *tolC*::Tn*5* (pBR322)	30
MC4100 *tolC*::Tn*10* Δ*acrAB*	40
MC4100 *thr*::Tn*10* Δ*acrAB*	40
AG100 *thr*::Tn*10*	200
AG100A *thr*::Tn*10*	40

[a]The susceptibilities of the indicated strains were assessed as indicated in the Materials and methods section. The results are the average of two or more assays.

the MIC of tetracycline for MC4100 *tolC*::Tn*10* and several *tolC* mutants carrying the Tn*10* in other sites on the chromosome (*thr*::Tn*10*). It can be seen in Table 2 that while the chromosomal copy of Tn*10* provides the expected high-level tetracycline resistance in the control strain MC4100 *thr*::Tn*10* (200 mg/L), it confers 5-fold lower levels (40 mg/L) of tetracycline resistance in the *tolC* derivatives. The experiments with the *tolC thr*::Tn*10* strains seemed to discard a polar effect of the *tolC*::Tn*10* insertion on a downstream gene. To confirm the role of the TolC protein itself in tetracycline resistance, plasmid pAX629, which carries a 1.9 kb chromosomal DNA fragment encoding the TolC protein only,[17] was introduced into the MC4100 *tolC*::Tn*10* strain. The transformants fully recovered the high tetracycline resistance levels (Table 2). Taken together, these results led us to conclude that *tolC* mutations somehow compromise Tet(A)-mediated resistance.

High-level resistance of constitutive tet(A) genes encoded by plasmids is also affected by tolC mutations

As described above, in TolC$^-$ cells expressing the single-component Tet(A) efflux pump of Tn*10*, high-level resistance to tetracycline is severely compromised. We wished to know whether this also occurred with another class of Tet(A) protein, that expressed constitutively from plasmid pBR322. MC4100 and MC4100 *tolC*::Tn*5*/*10* cells were transformed with this plasmid and their resistance to tetracycline was measured. As shown in Table 2, in a *tolC* background the highest level of tetracycline resistance conferred by the plasmid was 30 mg/L, as compared with 180 mg/L for the control. Thus, the efficiency of the pBR322-encoded Tet(A) pump was also reduced by the *tolC* mutation. Similar results were obtained with plasmid pACYC184, which carries the same tetracycline resistance gene as pBR322 (results not shown).

tolC::Tn10 mutants are also hypersusceptible to chlortetracycline

During the course of our study we attempted to generate a *tolC* deletion mutant from strain MC4100 *tolC*::Tn*10*. To achieve this we used the technique developed by Bochner *et al*.,[18] which is based on the finding that fusaric acid kills tetracycline-resistant cells in which the tetracycline resistance gene of the transposon Tn*10* has been induced by autoclaved chlortetracycline (50 mg/L). Note that when chlortetracycline is autoclaved in broth it is denatured so as to lose its toxicity towards tetracycline-susceptible cells while retaining its inducing ability for tetracycline-resistant cells.[18] Despite repeated attempts, we did not succeed in obtaining tetracycline-susceptible clones. We reasoned that the failure could be due to either fusaric acid or autoclaved chlortetracycline being toxic to the tetracycline-susceptible *tolC* deletion mutants we were seeking (the cells that had lost the transposon would probably still be *tolC*, since this leaves at high frequency a genetic lesion at the site where the Tn*10* was inserted). We therefore tested well-characterized *tolC* mutants in which the gene has been inactivated by Tn*5* insertion (MC4100 *tolC*::Tn*5*), deletion (PB3) or a point mutation (A586), and all of them proved susceptible to heated chlortetracycline at 50 mg/L. We concluded that the heated chlortetracycline in the selective medium was responsible for the toxicity to the *tolC* mutants that otherwise would be generated. This came as a surprise, since heated chlortetracycline has been reported to be non-toxic to *E. coli* cells. In fact, the parent MC4100 was unaffected by the heat-detoxified antibiotic at 50 mg/L. Although TolC$^-$ strains were susceptible to heated chlortetracycline at 50 mg/L, they grew well at a concentration of 10 mg/L of the autoclaved antibiotic. A possible explanation is that autoclaving does not abolish chlortetracycline activity, and that the antibiotic accumulates within *tolC* cells until it reaches a toxic level. MC4100 *tolC*::Tn*10* did not grow in the presence of 10 mg/L of native chlortetracycline. In this regard, Traub and Beck[19] found that the level of resistance to chlortetracycline of an *E. coli* K-12 strain carrying Tn*10* was 42 mg/L (uninduced cells) or 65 mg/L (preinduced). Thus, chlortetracycline resistance is also compromised by loss of *tolC*.

tet(A) gene expression is not altered in tolC mutants

Mutations in *tolC* elevate the transcription of *micF* antisense RNA, resulting in the concomitant reduction of OmpF.[20] Likewise, the decreased resistance to tetracycline in *tolC* mutants could be the result of an effect on the level of expression of Tn*10* *tet*(A). Therefore, the influence of a *tolC* mutation on the expression of a *tet*(A)::*lacZ* gene fusion was examined. For these experiments, the medium-copy-number plasmid pRKH40,[21] harbouring a *tet*(A)::*lacZ* fusion whose transcription is under the control of the repressor TetR, was transformed into strains MC4100 and MC4100 *tolC*::Tn*5*. Expression of the fusion in the TolC$^-$ strain (1618 and 1629 Miller units in log and stationary phase, respectively) was similar to that in the TolC$^+$ parent strain (1283 and 1134 Miller units). This suggests that the effect of *tolC* mutations on tetracycline resistance was not due to a down-regulation of the *tet*(A) gene.

Tetracycline efflux and accumulation assays

Given the involvement of TolC with known efflux systems, the increased tetracycline and chlortetracycline susceptibilities probably result from a decrease in drug efflux. To test this possibility we examined the kinetics of tetracycline uptake and release by using a method which relies on the fact that internalized tetracycline becomes fluorescent, and once released from the cells it

can be quantified with a spectrofluorometer.[11,22] As shown in Figure 1, there was a slower efflux from the *tolC*::Tn*10* mutant than in the MC4100 *thr*::Tn*10* strain. Values for the *tolC* strain approached those for the parental, tetracycline-susceptible strain MC4100.

Since an impairment of tetracycline efflux would lead to more drug being retained within cells, we next investigated tetracycline accumulation levels in the same strains used for the efflux experiments. To this end, cells were preloaded with tetracycline (100 mg/L) during a 15 min incubation, pelleted and resuspended in tetracycline-free buffer. Under these conditions, intracellular fluorescent tetracycline is immediately released into the buffer and could be detected by a spectrofluorometer, providing a quantitative estimate of the amount of tetracycline accumulated during the 15 min loading phase. As can be seen in Figure 2(a), fully tetracycline-resistant MC4100 *thr*::Tn*10* cells accumulate the smallest amount of tetracycline, whereas the *tolC*::Tn*10* mutation resulted in significantly higher intracellular tetracycline levels, which were comparable to that observed in the susceptible MC4100 strain. When the *tolC*::Tn*10* strain was transformed with pAX629, which complements the *tolC* mutation, the accumulated antibiotic became closer to that of the *thr*::Tn*10* strain. In other experiments, the absolute amount of tetracycline accumulated was measured by its conversion into anhydrotetracycline, as described in the Materials and methods section. As shown in Figure 2(b), there is a precise correlation between the results obtained with this method and those of the fluorescence assay. In particular, the *tolC* mutant accumulated a higher level of tetracycline than did strain MC4100 *thr*::Tn*10*. Taken together, the above results are consistent with a defect in drug efflux in the *tolC*::Tn*10* mutant.

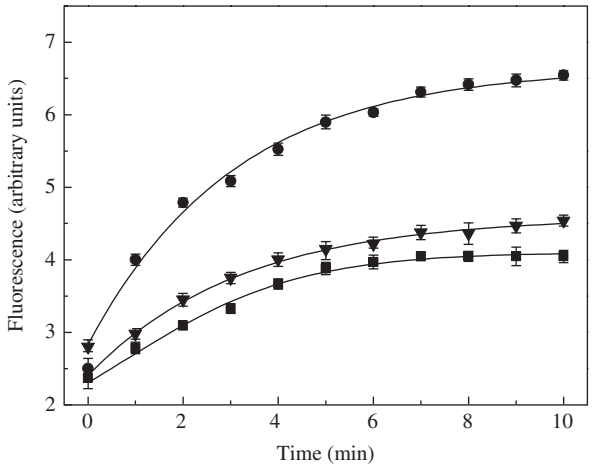

Figure 1. Tetracycline efflux. Bacteria were loaded with tetracycline and efflux measured using spectrofluorometry, as indicated in the Materials and methods section. Filled circles, MC4100 *thr*::Tn*10*; filled triangles, MC4100 *tolC*::Tn*10*; filled squares, MC4100.

Contribution of the AcrAB multidrug efflux system to high-level tetracycline resistance

That the operation of TolC involves drug efflux strongly supported the involvement of additional efflux components, since outer membrane proteins are unable to function alone in energy-dependent transport processes. We considered the possibility that TolC could be associated with the Tet protein, and probably with a linker protein connecting them. However, Thanassi *et al.*[23] found convincing evidence showing that the Tet protein pumps out tetracycline into the periplasm and not directly into the medium.

Mutations in *tolC* would lead to a non-functional AcrAB-TolC system. However, the AcrAB efflux mechanism confers a low level of resistance as compared with Tet(A). For example, the tetracycline MIC for a wild-type *E. coli* K-12 strain, such as W3110, is typically around 1.25 mg/L.[24] Thus, we thought it unlikely that the large decrease in MIC of tetracycline, from 200 to 40 mg/L, seen in *tolC* mutants carrying a Tn*10* could be accounted for by the loss of the AcrAB efflux activity caused by the mutation. We decided to assess the contribution of the AcrAB pump to high-level tetracycline resistance. The Δ*acrAB*::*kan* mutation from strain AG100A[25] was

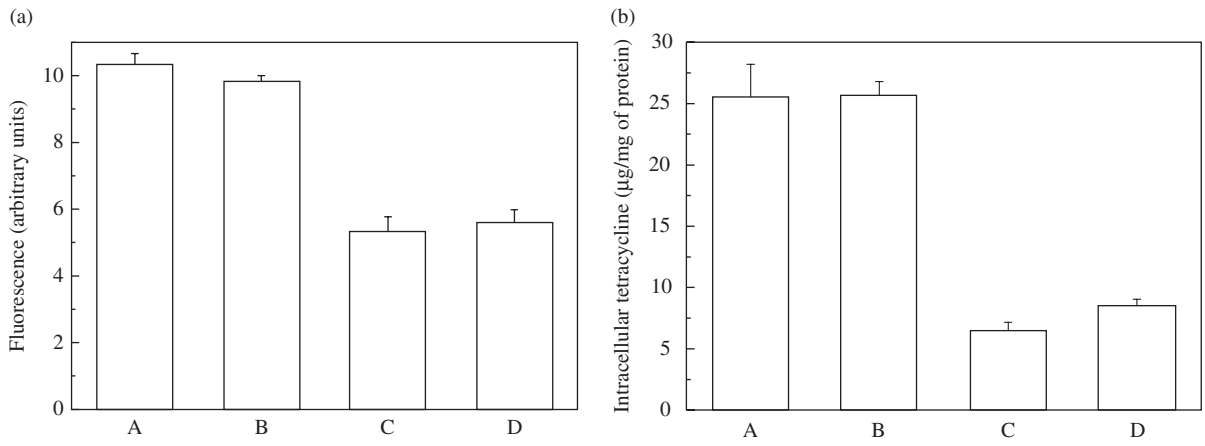

Figure 2. (a) Tetracycline accumulation measured using spectrofluorometry. Bacteria were loaded with tetracycline, pelleted and resuspended in Mg^{2+} buffer, in which released fluorescence (a direct measurement of accumulated tetracycline) was immediately detected with a spectrofluorometer. The bars represent the following: A, MC4100; B, MC4100 *tolC*::Tn*10*; C, MC4100 *thr*::Tn*10*; D, MC4100 *tolC*::Tn*10* (pAX629). Values are the means of at least six independent determinations. Standard deviations are shown. (b) Total accumulation of tetracycline determined after conversion into anhydrotetracycline. The bars represent the same strains as in panel (a). Intracellular tetracycline is expressed as μg of antibiotic/mg of protein. Experiments were repeated six times with almost identical results. Error bars indicate standard deviations.

transduced into MC4100 *thr*::Tn*10* with phage P1vir, selecting for kanamycin resistance. Interestingly, the tetracycline MIC for the resultant transductant, defective in the AcrAB pump, dropped from 200 mg/L in the parent strain to 40 mg/L, the same value observed for the MC4100 *thr*::Tn*10 tolC* mutant. The converse experiment was also performed. The *thr*::Tn*10* mutation was transferred to strains AG100 and AG100A, and the tetracycline resistance of the transductants was tested. As shown in Table 2, a similar reduction in tetracycline resistance was observed. That the *acrAB* and *tolC* mutations, in otherwise isogenic strains, reduced tetracycline resistance to the same extent indicates that the mutations act through a mutual mechanism. By searching in the literature, we noticed a paper by Lee *et al*.[26] which could provide a possible explanation to our results. These authors showed that when a single-component efflux pump and a multi-component efflux pump with shared substrates are co-expressed in the same cell, the observed antibiotic resistance is much higher than that conferred by each of the pumps expressed singly. Moreover, recent functional[27–29] and structural[30–32] data favour the view that for multi-component efflux pumps, exemplified by the *E. coli* AcrAB system, the substrate capture may occur in the periplasm. Altogether, our results suggested that the single-component Tet(A) transports tetracycline from the cytosol to the periplasmic space and the AcrAB multi-component pump promotes efflux from this space to the external medium, resulting in a multiplicative enhancement of the level of drug resistance. At tetracycline concentrations of 40 mg/L or lower, in the absence of a functional AcrAB-TolC pump, the action of Tet(A) and the rapid exit of tetracycline extruded into the periplasm through the OmpF porin would be sufficient to balance the re-entry of tetracycline back into the cytoplasm. However, at higher external tetracycline concentrations this mechanism could be overrided and Tet(A) should have to work in combination with AcrAB to ensure high levels of resistance.

Lee *et al*.[26] have observed a multiplicative level of tetracycline resistance in *Pseudomonas aeruginosa* by combining Tet(A) and the multidrug efflux system MexAB–OprM (MexAB is an AcrAB homologue). In the present work, starting by a chance observation that a strain with a Tn*10* insertional inactivation of *tolC* gave smaller colonies on tetracycline plates, we experimentally validated for the first time that a similar mechanism may be operational in *E. coli*.

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Transparency declarations

None to declare.

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