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RESEARCH

Engineering Biological Approaches for Detection of Toxic Compounds: A New Microbial Biosensor Based on the *Pseudomonas putida* TtgR Repressor

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Abstract Environmental contamination by toxic organic compounds and antimicrobials is one of the causes for the recent surge of multidrug-resistant pathogenic bacteria. Monitoring contamination is therefore the first step in containment of antimicrobial resistance and requires the development of simple, sensitive, and quantitative tools that detect a broad spectrum of toxic compounds. In this study, we have engineered a new microbial biosensor based on the *ttgR*-regulated promoter that controls expression of the TtgABC extrusion efflux pump of *Pseudomonas putida*, coupled to a *gfp* reporter. The system was introduced in *P. putida* DOT-T1E, a strain characterized by its ability to survive in the presence of high concentrations of diverse toxic organic compounds. This whole-cell biosensor is capable to detect a wide range of structurally diverse antibiotics, as well as compounds such as toluene or flavonoids.

Keywords Antimicrobial resistance · Microbial biosensor · *Pseudomonas* · Extrusion efflux pump · TtgR repressor

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Introduction

Bacterial resistance to antibiotics and other therapeutic agents is an increasingly worrisome phenomenon in medicine. Unnecessary and excessive use of antimicrobials, as well as their release into the environment, has caused what some authors already call an "antibiotics crisis" [1]. A recent report from the US Centers for Disease Control and Prevention [2] reflects the increasing menace of infection by antibiotic-resistant bacteria on human health, with an estimate of around 23,000 deaths per year in the US attributable to such infections. The World Health Organization published in 2001 a global strategy for containment of antimicrobial resistance, setting some recommendations to minimize the effects of this problem. These included two important steps: limiting the use of antimicrobials and promoting the discovery of new anti-infectives. In a recent publication, the need for surveillance of antimicrobial resistance and use is also included as an essential control element [3].

Chromatographic techniques or antibiogram tests have been established to detect specific antibiotics. However, these techniques provide limited information and sometimes require tedious sample pre-treatments [4–7]. Nowadays, the emerging technology is oriented to the development of microbial biosensors: analytical devices which integrate microorganism(s) with a physical transducer to generate a measurable signal, proportional to the concentration of analytes [8]. Assays based on biosensors show crucial advantages over other techniques, including high sensitivity, reliability, and simplicity of operation [9–15]. Microbial biosensors can also be engineered to be highly specific or to sense a broad spectrum of antibiotics, unlike other techniques that detect particular antibiotic structures [16]. Whole-cell biosensors, which produce

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measurable gene products encoded by reporter genes, are among the most useful. They can be classified as general, specific, or semi-specific biosensors. General biosensors have been described as those containing a reporter gene placed downstream of a constitutively expressed promoter, reporting a decrease in metabolic activity reflected in a lower signal from the reporter. Specific biosensors include a chimeric construction that contains a reporter gene fused to a regulated promoter and in some cases, to the protein responsible for the activation/repression of the promoter in response to particular compounds or conditions. Finally, semi-specific biosensors are those in which the reporter gene is fused to a stress-responsive promoter whose expression occurs in response only to certain compounds or conditions that cause stress to the cell [17].

In this work, we report the development of a simple, quantitative, and sensitive microbial biosensor for the detection of a range of antimicrobials at low concentrations. We have made use of *Pseudomonas putida* DOT-T1E as a microbial system, given its metabolic versatility, environmental adaptability, and high resistance to toxic organic compounds [18–22]. The biosensor is based on the green fluorescence protein (GFP) as reporter, fused to the promoter of the *ttgABC* operon, which encodes an efflux pump involved in resistance to antibiotics and aromatic compounds whose expression is activated by such molecules via the transcriptional repressor TtgR.

Materials and Methods

Bacterial Strains and Growth Conditions

Pseudomonas putida DOT-T1E [18], a strain originally isolated from a water treatment plant, was used as the basis for the microbial biosensor, and *Escherichia coli* DH5α was used in cloning experiments [23]. Both strains were routinely grown in Luria–Bertani broth (LB) [24] at 30 °C or 37 °C, respectively.

Cloning Techniques

To construct the biosensor, a 257 bp fragment containing the *ttgABC-ttgR* intergenic region from *P. putida* DOT-T1E was amplified using primers GST-ttgABC-R-fw (5́-AAACCCAAGCTTGGGTAAGCTAATCAAGCGCTG AGGCTGTACCTG-3́) and GST-ttgABC-R-rv (5́-CATG AGGATCCTCGGGTCGCTGGAG-3́). The resulting PCR product was digested with HindIII and BamHI and cloned into plasmid pRU1097 [25] cut with the same enzymes, upstream of the promoterless *gfp* gene (gfpmut3.1) (Fig. 1). The resulting recombinant construction, pMMCL, was electroporated into *P. putida* DOT-T1E.

Fluorescence Measurements

Growth and fluorescence of the biosensor in the presence of different molecules was measured in a Cary Eclipse spectrofluorimeter (Varian Inc.). Measurements were done at $\lambda_{excitaton} = 450$ nm and $\lambda_{emission}$ interval = 500–600 nm. Background fluorescence from the growth medium was subtracted and the average relative fluorescence with respect to the negative control was then calculated from the obtained data at three λ intervals (519–521, 526–528 and 532–534 nm). Graphs correspond to the average relative fluorescence and standard errors of three independent experiments.

Results and Discussion

Construction of the ttgR-Based Biosensor

Many *P. putida* strains show high resistance against aromatic and xenobiotic compounds as a result of the active extrusion of toxic compounds through membrane-bound efflux pumps regulated at the transcriptional level [26]. Particularly, *P. putida* DOT-T1E is characterized by its ability to survive in the presence of high concentrations of diverse organic compounds such as toluene, flavonoids, β-lactamic antibiotics, and other antimicrobials [18–22]. The primary contributor to resistance to this set of toxic chemicals is the TtgABC efflux pump, regulated by TtgR, a member of the TetR family of transcriptional repressors [27]. Previously, TtgR has been described as the only reporter repressor that binds to different antibiotics, flavonoids, and organic solvents which are also extruded by the efflux pump [28–30]. The transcriptional regulator TtgR binds to the *ttgABC-ttgR* intergenic region, which contains the overlapping divergent promoters for *ttgABC* (P_{ttgABC}) and *ttgR* (P_{ttgR}) (Fig. 1a). In the absence of effectors, TtgR inhibits transcription of the *ttgABC* operon and controls its own expression. Derepression takes place when a substrate binds to the active binding site of TtgR and the repressor is released from its cognate operator.

This system was used to design and construct a biosensor. As described in Materials and Methods (see Sect. 2.2.), a DNA fragment containing the *ttgABC-ttgR* intergenic region from *P. putida* DOT-T1E was amplified and cloned into the promoter-probe plasmid pRU1097 [25]. The resulting recombinant construction, pMMCL, was selected by gentamicin resistance which does not increase the basal levels of expression of *ttg*ABC and *ttg*R promoters [27] avoiding any interference. Once electroporated into DOT-T1E, green fluorescence resulting from expression of GFP in the recombinant construction, pMMCL, would be controlled by the presence of toxic compounds through the

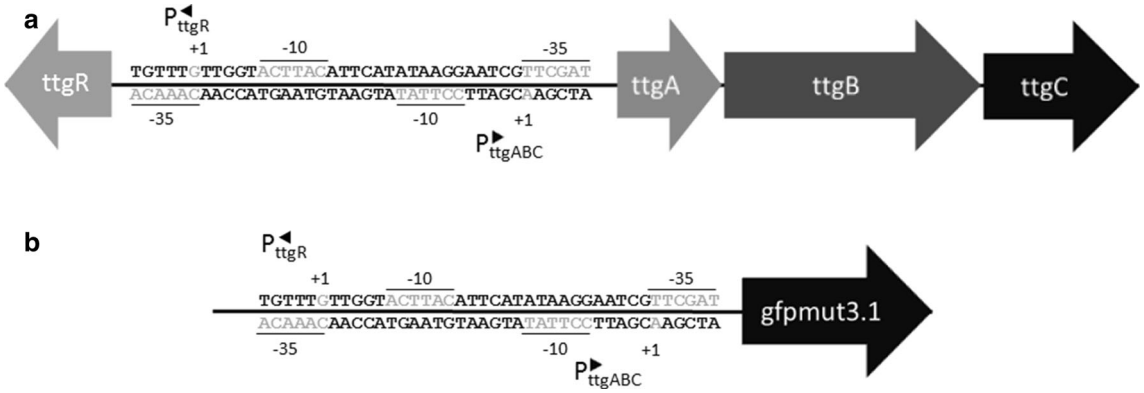

Fig. 1 **a** Nucleotide sequence of *ttg*R-*ttg*ABC intergenic region. The position +1, −10, and −35 boxes of the divergent promoters are marked in *light grey*. **b** Schematic representation of the construction introduced in pRU1097 expression vector

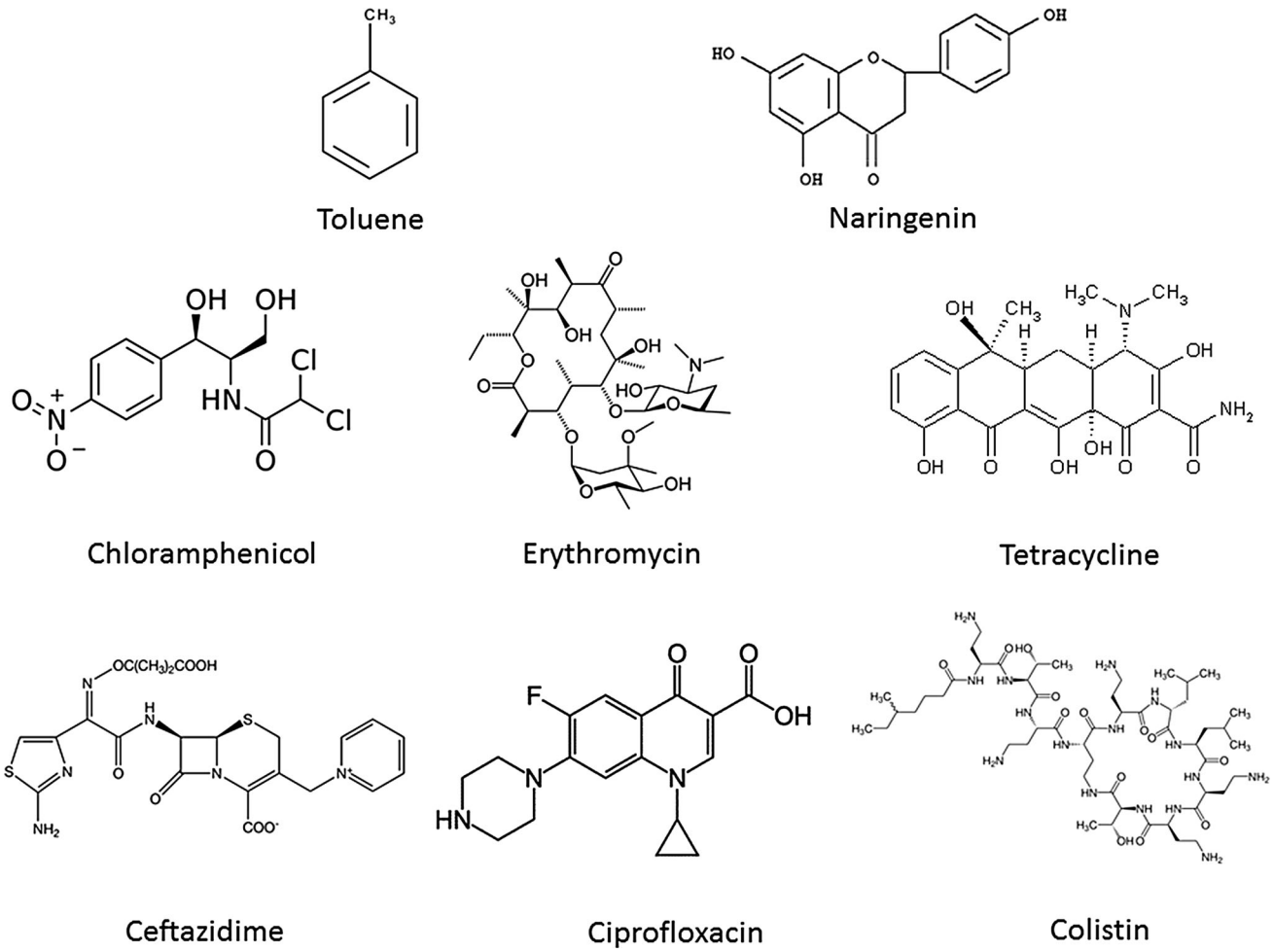

Fig. 2 Chemical structural formulas of the compounds tested by the microbial biosensor

ttgR regulatory gene product. The functionality of the biosensor was first tested in *P. putida* grown on LB-agar plates at 30 °C, in the absence and in the presence of a saturated atmosphere of toluene provided in the gas phase by means of a hollow glass rod containing filter paper impregnated in toluene. Green fluorescence was only observed under UV light in the case of *P. putida* DOT-T1E (pMMCL) grown in the presence of toluene, indicating that the biosensor is responding to this toxic compound, as expected (Supplementary material Fig. S1).

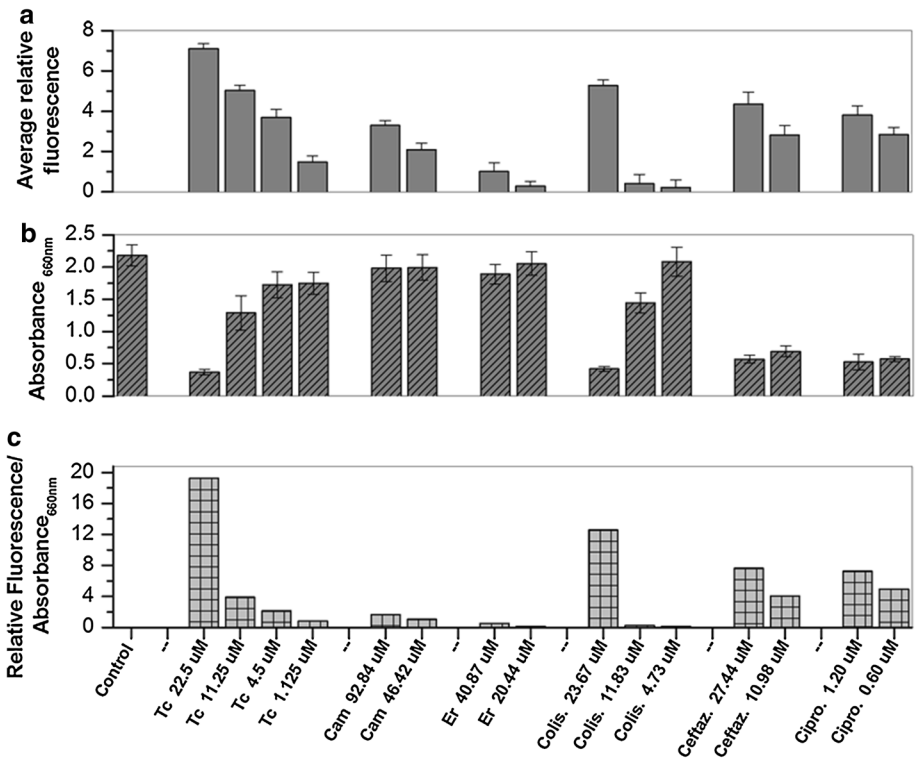

Fig. 3 **a** Graphical representation of the average relative fluorescence with respect to the each negative control in response to different concentrations of antibiotics. Tetracycline (Tc), Chloramphenicol (Cam), Erythromycin (Er), Colistin (Colis.), Ceftazidime (Ceftaz.) and Ciprofloxacin (Cipro.). **b** Optical density graph at 660 nm of wavelength of the bacterial cultures after 1 day of growing in the presence of several concentrations of antibiotics and the control in absence of any antibiotics. **c** Comparison of the Relative fluorescence to the optical density at 660 nm at all of the antibiotics concentrations tested

Biosensor Response to Antibiotics

The sensitivity and range of substrates to which the biosensor responds was examined by spectrofluorometry as described below. *P. putida* DOT-T1E harboring pMMCL was grown in liquid LB at 30 °C and 200 rpm of shaking. After 2 h of growth, the cultures were supplemented with different effectors and allowed to grow for 24 h. Fluorescence was then measured spectrofluorometrically.

Six different antibiotics were selected for this assay: chloramphenicol (Cam), erythromycin (Er), tetracycline (Tc), ceftazidime (Ceftaz.), ciprofloxacin (Cipro.), and colistin (colis) (Fig. 2).

Three of them are specific for clinical use against *Pseudomonas.* Ceftazidime is a third-generation cephalosporin antibiotic with a broad spectrum activity against Gram-positive and Gram-negative bacteria [31]. It is active against *Pseudomonas aeruginosa* but shows weaker activity against Gram-positive microorganisms. Ciprofloxacin is a second-generation fluoroquinolone antibiotic [32]. It has a broad spectrum of activity that includes Gram-negative bacteria as *E. coli, Haemophilus influenza, Legionella pneumophila*, *P. aeruginosa* etc. and also includes Gram-positive bacteria as *Staphylococcus aureus* etc. Colistin is produced by *Bacillus polymyxa* strain var. *colistinus* [33]. It is a mixture of cyclic polypeptides; colistin A and B. It is effective against most Gram-negative bacilli. Tetracycline, erythromycin, and chloramphenicol act inhibiting the bacterial protein synthesis. Tetracycline acts as bacteriostatic at usual doses, against Gram-positive and Gram-negative bacteria with the exception of *P. aeruginosa,* but at high doses is bactericidal. On the contrary, erythromycin is a specific antibiotic of Gram-positive bacteria.

The spectrofluorometric results (Fig. 3a) showed different fluorescence intensities according to the sample concentration tested. So, the highest intensity of fluorescence in respect to the lowest concentration used corresponds to Ciprofloxacin followed by tetracycline. The lowest intensity was measured for erythromycin. The intensity decay for tetracycline correlated with the decrease of the antibiotic concentrations tested. On the other hand, chloramphenicol resulted in very low fluorescence intensity except at the higher antibiotic concentrations tested. Because of the low sensitivity of the biosensor, it was not possible to sense chloramphenicol concentrations below 30 μM.

The sensor responded well to colistin at a concentration around 20 μM showing abrupt intensity decay at lower concentrations. Colistin is one of the last-resort antibiotics for multidrug-resistant *P. aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter.* It is a surfactant that alters the permeability of the bacterial cell membrane leading to cell death. This strong antimicrobial effectiveness could be the reason of the abrupt fluorescence change by decreasing of concentration. The biosensor also responded to ceftazidime and ciprofloxacin in a dose-dependent manner but the

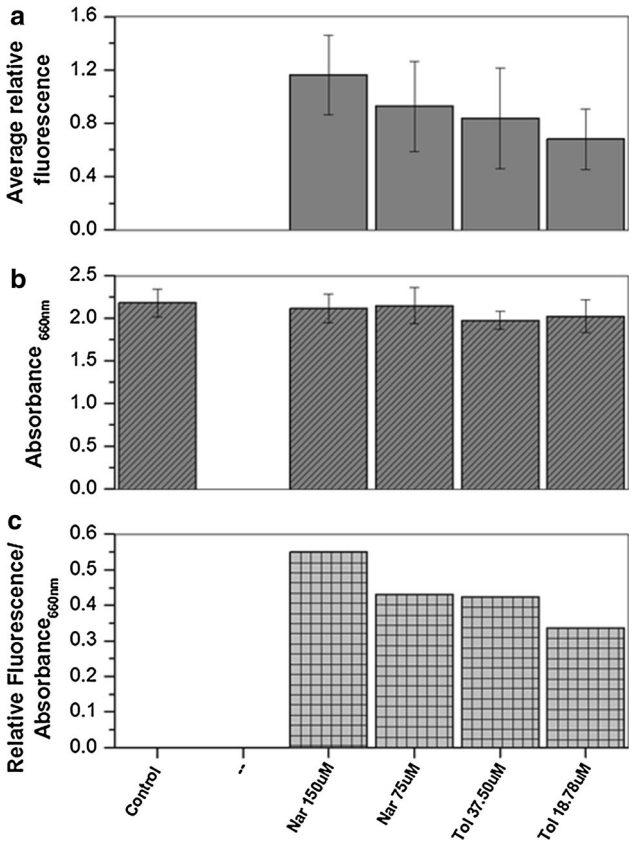

Fig. 4 **a** Graphical representation of fluorescence response, of the engineered GFP system, in *Pseudomonas putida* DOT-T1E upon addition of several concentrations of effectors. Two first bars correspond to Naringenin (Nar) at 150 and 75 μM and the last ones to toluene (Tol) at 37.5 and 18.78 μM. **b** Optical density graph at 660 nm of wavelength of the bacterial cultures after 1 day of growing in the presence of two concentrations of naringenin and toluene and the control in absence of any effector. **c** Comparison of the Relative fluorescence to the optical density at 660 nm at all of the antibiotics concentrations tested

sensitivity for each compound was very different. Ceftazidime is a bactericide that inhibits bacterial cell wall synthesis and is stable in the presence of clinically relevant beta-lactamases. Ciprofloxacin is a bactericide that interferes with bacterial DNA replication by inhibiting the DNA gyrase and bacterial topoisomerase IV. Both antibiotics have a broad activity spectrum.

To determine the influence of the different compounds tested on bacterial growth and survival, the turbidity of the cultures was also measured by optical density at 660 nm (Fig. 3b). The absorbance obtained for each sample was corrected by subtraction of its corresponding negative control. The most significant bacterial growth limitation, at the lowest antibiotic concentrations, was detected for ciprofloxacin followed by ceftazidime, tetracycline, and colistin around 22 μM. While chloramphenicol and erythromycin, at higher concentrations, did not have a significant influence on bacterial growth. Since the bacterial culture growth is not the same in the presence of each antibiotic and to present a more clear view of the effect of the growth on the fluorescence measure we have represented the relative fluorescence respect to the absorbance at 660 nm (Fig. 3c). We can conclude that the biosensor is able to sense any of the antibiotics tested being most sensible to ciprofloxacin followed by tetracycline and ceftazidime and the less sensible to chloramphenicol and erythromycin.

Biosensor Response to Other Toxic Compounds

The response of the biosensor to other compounds was also tested in liquid cultures. Flavonoids are a class of secondary metabolites secreted by plant roots as defence molecules with antimicrobial properties. It has been demonstrated that TtgR protein binds several flavonoids, such as naringenin, with different specificities [34]. We tested two different concentrations of naringenin (150 and 75 μM). The biosensor allowed detecting low concentrations of naringenin, being the higher value of average relative fluorescence for 150 μM (Fig. 4a).

In the case of toluene, the fluorescence signal increased with the concentration of the aromatic hydrocarbon (Fig. 4a), as in the case of naringenin. However, the concentrations tested were much lower to minimize growth limitations caused by toluene (Fig. 4b). The comparison of fluorescence intensities relative to culture growth (Fig. 4c) showed that toluene at a concentration of 37.5 μM gave the same response as naringenin at twice that concentration, indicating higher sensibility of the biosensor to toluene than to the flavonoid.

Conclusions

The goal of this study was the design, construction, and development of a simple biosensor capable to sense low concentrations of a broad spectrum of toxic compounds, by means of the TtgR transcriptional regulator. In all cases, we observed a dose-dependent response, with an average relative fluorescence rise upon increasing concentrations of the compounds added. The average relative fluorescence values obtained for toluene, naringenin, and erythromycin were smaller than those obtained for the other compounds. This could suggest that, although TtgR has been usually studied from the perspective of toluene tolerance, this regulator is able to either bind or indirectly respond to a wide range of unrelated toxic compounds. Testing natural antimicrobial compounds would therefore be one of the relevant uses of this biosensor. The results also open the way to construct versions of the biosensor that are specific

for certain compounds, by mutating the binding site of TtgR and screening for different toxic compounds.

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Conflict of interest The authors declare no conflicts of interest.

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