environmental microbiology
environmental microbiology reports

Interspecies signaling: Pseudomonas putida efflux pump TtgGHI is activated by indole to increase antibiotic resistance

Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID:	EMI-2013-1213
Manuscript Type:	EMI - Research article
Journal:	Environmental Microbiology
Date Submitted by the Author:	29-Sep-2013
Complete List of Authors:	Molina-Santiago, Carlos; Estación Experimental del Zaidín-CSIC, Daddaoua, Abdelali; Estación Experimental del Zaidín-CSIC, Fillet, Sandy; CSIC - EEZ, Proteccion Ambiental Duque, Estrella; EEZ /CSIC, Bioquimica Ramos, Juan; CSIC, Estacion Experimental del zaidin; CSIC, Estacion Exp. del Zaidin;
Keywords:	community genetics, ecophysiology, environmental signal/stress responses, microbe:microbe interactions

Interspecies signaling: *Pseudomonas putida* efflux pump TtgGHI is activated by indole to increase antibiotic resistance

Carlos Molina-Santiago[1], Abdelali Daddaoua[1], Sandy Fillet[1,δ], Estrella Duque[1], and Juan-Luis Ramos[1*,□]

[1] Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, Profesor Albareda 1, 18008 Granada, Spain

[δ] Current address: Neuron Biopharma, PTS, Armilla, Granada, Spain

□ Current address: Abengoa Research/Loyola University, Sevilla, Spain

*Corresponding author: Juan Luis Ramos

Abengoa Research/Loyola University Center

Campus Palmas Altas

41014 Sevilla, Spain

Phone:

e-mail: ramosmartin1956@gmail.com

Summary

In Gram-negative bacteria multidrug efflux pumps are responsible for the extrusion of chemicals that are deleterious for growth. Some of these efflux pumps are induced by endogenously produced effectors while abiotic or biotic signals induce the expression of other efflux pumps. The TtgABC efflux pump is the main antibiotic extrusion system that respond to exogenous antibiotics through the modulation of the expression of this operon mediated by TtgR. The plasmid-encoded TtgGHI efflux pump in *Pseudomonas putida* plays a minor role in antibiotic resistance in the parental strain; however, its role is critical in isogenic backgrounds deficient in TtgABC. Expression of *ttgGHI* is repressed by the TtgV regulator that recognizes indole as an effector, although *P. putida* does not produce indole itself. Since indole is not produced by *Pseudomonas* the indole-dependent antibiotic resistance seems to be part of an antibiotic resistance programme at the community level. *Pseudomonas putida* recognizes indole added to the medium or produced by *Escherichia coli* in mixed microbial communities. Transcriptomic analyses revealed that the indole-specific response involves activation of 43 genes and repression of 23 genes. Indole enhances not only the expression of the TtgGHI pump, but also a set of genes involved in iron homeostasis, as well as genes for amino acid catabolism. In a *ttgABC* deficient *P. putida* background ampicillin and other bactericidal compounds leads to cell death. Co-culture of *E. coli* and *P. putida ttgABC* allowed growth of the *P. putida* mutant in the presence of ampicillin due to induction of the indole-dependent efflux pump.

Pseudomonas putida are ubiquitous microorganisms that can be found in soils associated with plant roots and in aquatic systems either in suspension or in biofilms on biotic and abiotic surfaces (Yousef-Coronado *et al.*, 2008; Rodríguez-Herva *et al.*, 2010; Jakovleva *et al.*, 2012). Survival and proliferation under diverse environmental conditions is based on an ample interplay of metabolic activities and the ability of bacteria of this species to respond to antimicrobial compounds produced by other microorganisms (Daniels and Ramos, 2009).

Pseudomonas putida strain DOT-T1E was isolated from the Granada wastewater treatment plant as a bacterium able to thrive in the presence of high concentrations of organic solvents such as aromatic compounds (Ramos *et al.*, 1995). Since then, DOT-T1E has been shown to be resistant to a wide range of toxic compounds such as dyes, heavy metals and a broad array of bactericidal antibiotics, e.g., ampicillin, piperacillin, norfloxacin, and bacteriostatic compounds such as chloramphenicol, tetracycline and erythromycin (Ramos *et al.*, 1998; Terán *et al.*, 2003; Fernández *et al.*, 2012). The main mechanism of resistance towards bactericidal and bacteriostatic antibiotics in this microorganism is extrusion from the cell to the outer medium through the action of a number of multidrug efflux pumps of the resistance-nodulation-division (RND) family (Rojas *et al.*, 2001). We have identified 19 RND efflux pumps in the genome of *P. putida* DOT-T1E (Udaondo *et al.,* 2012 and Suppl. Table 1), two of which have been experimentally shown to be involved in antibiotic extrusion. One of these pumps, TtgABC (T1E_0241-0243) is chromosomally located (Rojas *et al.*, 2001) and is considered the most relevant efflux pump involved in resistance to antibiotics in this strain (Duque *et al.*, 2001; Terán *et al.*, 2007; Roca *et al.*, 2008); the other efflux pump to which a role has been assigned in antibiotic resistance is the plasmid-encoded TtgGHI efflux pump, which is most relevant in solvent extrusion (Mosqueda and

Ramos, 2000). The expression of the TtgABC pump takes place at a basal level and it is further controlled by the TtgR regulator, a member of the TetR family in response to certain antibiotics and flavonoids (Duque *et al.*, 2001; Ramos *et al.*, 2005). The expression of the TtgGHI efflux pump is controlled by the repressor TtgV, a member of the IclR family (Molina-Henares *et al.*, 2006) and indole, a molecule that is not synthesised by *Pseudomonas*, and that has been reported to act as an efficient effector for TtgV.

Indole has been proposed to act as an intra and intercellular signalling molecule (Lee and Lee, 2010; Han *et al.*, 2011). We hypothesized that these two efflux pumps could play differential roles in antibiotic resistance with one of them (TtgABC) being part of the cell's own self-resistance at the single cell level, while the one induced by indole (TtgGHI) could be part of antibiotic resistance at the community level. In enteric bacteria indole produced from tryptophan through the action of tryptophanase (Yanofsky *et al.*, 1991), is not metabolized by the cells and is excreted to the outer medium, where it can reach concentrations as high as 0.5 mM (Wang *et al.*, 2001; Kobayashi *et al.*, 2006). Indole from the outer medium is also taken up by *Escherichia coli* cells and is involved in the control of numerous processes such as drug resistance (Hirakawa *et al.*, 2005; Lee and Lee, 2010), plasmid stability (Chant and Summers, 2007), virulence traits in certain pathogenic strains (Anyanful *et al.*, 2005; Hirakawa *et al.*, 2009; Chu *et al.*, 2012) and biofilm formation (Martino *et al.*, 2003; Lee *et al.*, 2007). Therefore, indole acts as an intra-species signal molecule. Conversely, indole can also be taken up by non-enteric bacteria and its uptake also influences a number of phenotypes such as inhibition of growth in for example *Aspergillus niger* (Kamath and Vaidyanathan, 1990), enhanced drug resistance in *Salmonella enterica* (Nikaido *et al.*, 2008; Vega *et al.*, 2013), favours biofilm formation in *Pseudomonas aeruginosa*,

Pseudomonas fluorescens and *Burkholderia unamae* (March and Bentley, 2004; Lee *et al.*, 2007; Ueda and Wood, 2009; Vert and Chory, 2011; Kim *et al.*, 2013); indole also attenuated virulence in *Pseudomonas aeruginosa* (Lee *et al.*, 2009). Therefore, indole is also an interspecies signal molecule.

In this study we explore the response of different isogenic *P. putida* strains to bactericidal and bacteriostatic antibiotics, in the presence and absence of indole. We have also used *P. putida* as a model system to determine the role of indole as an interspecies signal. Indole influences the pattern of gene expression of at least 66 genes in *Pseudomonas putida*, these genes are involved in cell metabolism, oxidative stress and metal transport; these results support indole's role as a signalling molecule in *Pseudomonas putida*. In this study we show that indole produced by enterobacteria enables *Pseudomonas* to thrive in the presence of drugs through the induction of the TtgGHI efflux pump and facilitation antibiotic extrusion.

RESULTS

Response of *P. putida* to bactericidal and bacteriostatic antibiotics analysed using fluorescent dyes

In *Pseudomonas putida* strain DOT-T1E the main mechanism of extrusion of certain bactericidal (ampicillin, norfloxacin) and bacteriostatic compounds (chloramphenicol, tetracycline and erythromycin) is achieved through the action of RND efflux pumps (Mosqueda and Ramos, 2000; Rojas *et al.*, 2001; Godoy *et al.*, 2010; Fernández *et al.*, 2012). In this study we have monitored cell growth arrest, cell death and 3'-*p*-(hydroxyphenyl) fluorescein (HPF) fluorescence quenching in the wild type *P. putida* DOT-T1E, and its isogenic mutants in with knock-outs at *ttgABC* (T1E-18), *ttgGHI* (T1E-PS28) and in the double mutant *ttgABC*/*ttgGHI* (T1E-PS32) (Table 1)

after exposure to ampicillin and norfloxacin (bactericidal compounds) and chloramphenicol, erythromycin and tetracycline (bacteriostatic antibiotics).

Firstly, we determined the MIC concentrations of the four strains for the above five antibiotics. We found that the MIC concentrations in T1E-PS28 were identical to those of T1E wild-type (Table 2), whereas T1E-18 and T1E-PS32, both deficient in TtgABC, exhibited increased susceptibility to antibiotics (Table 2). With bacteriostatic compounds we found that for the wild-type 300 µg/ml chloramphenicol, 400 µg/ml erythromycin or 8 µg/ml tetracycline halted cell growth, although 100% of the cells remained viable throughout the assay (3 h) (See Figure 1A for chloramphenicol and Suppl. Figure 1). For the TtgABC mutant, 70 µg/ml chloramphenicol sufficed to inhibit growth while even lower concentrations inhibited the growth of the double mutant (Table 2) although cells survived during the assay.

With bactericidal compounds we found that these type of antibiotics first stop cell growth and after 3h the number of viable cells decreased by 2 orders of magnitude due to cell lysis. For the parental strain DOT-T1E and the PS28 strain ampicillin at 625 µg/ml and norfloxacin at 2 µg/ml exerted the described effect. Growth of T1E-18 and T1E-PS32 was halted by even lower concentrations of these two bactericidal compounds, which was again followed by cell death.

Indole is an effector of TtgV, the repressor that modulates the expression of *ttgGHI*. We reasoned that in the presence of indole antibiotic resistance could be enhanced in *P. putida* DOT-T1E since the TtgGHI pump appears to have a role in antibiotic extrusion (Mosqueda and Ramos, 2000). We repeated the set of MIC assays with the parental strain and the *ttgABC*, *ttgGHI* and *ttgABC*/*ttgGHI* mutants in the presence of indole (See Table 2). We observed that the pattern of antibiotic resistance in DOT-T1E and T1E-PS28 (*ttgGHI* mutant) in the presence of indole was similar and did

not vary with respect to that found in the absence of indole (Table 2). The pattern of antibiotic resistance in the T1E-PS32 double mutant with and without indole revealed that the mutant was less resistant than the parental one to the set of tested antibiotics regardless of the presence of indole. However, the T1E-18 strain with indole exhibited enhanced resistance to antibiotics, which suggests that in the absence of TtgABC, the indole-dependent induction of TtgGHI is relevant in antibiotic resistance.

In the HPF quenching assays we monitored HPF fluorescence 3 h after addition of 50% of the MIC drug concentrations, a concentration and timing that guaranteed 100% survival of wild-type and mutant cells. We found that bacteriostatic compounds, such as chloramphenicol (Figure 1B), erythromycin and tetracycline (Suppl. Figure 1C and 1E) failed to promote HPF quenching in *P. putida* and its mutants, a result which matches those for *Escherichia coli*. For the wild type DOT-T1E with ampicillin (Figure 1C) and norfloxacin (Suppl. Figure 1G) we found that HPF quenching occurred. We also examined the response of HPF fluorescence quenching in T1E-18, T1E-PS28, and the double mutant T1E-PS32. We found that for DOT-T1E-18 concentrations as low as 100 µg ampicillin/ml were enough to induce the quenching reaction (Figure 1I). This value is 3-fold lower than the minimal concentration necessary to quench the signal in the wild-type. With the double mutant T1E-PS32 even lower concentrations (i.e., 15-fold) sufficed to induce the HPF fluorescence quenching (Figure 1L). This set of results indicated that although the role of the TtgGHI efflux pump in ampicillin extrusion is minor based on the pattern of HPF quenching and the profile of antibiotic resistance of the parental strain and mutant T1E-PS32 (Fig. 1), a role in ampicillin efflux should be ascribed to TtgGHI since in the double *ttgABC/ttgGHI* mutant HPF fluorescence quenching occurred at much lower ampicillin concentrations than in T1E-18 and the double mutant exhibited increased sensitivity to a range of antibiotics.

Bactericidal compounds do not kill *Pseudomonas putida* DOT-T1E through the formation of reactive oxygen species.

Setsukinai *et al.* (2003) showed that *in vivo* hydroxyl radicals quenched HPF fluorescence. Kohanski *et al.* (2007) suggested a universal killing mechanism in *E. coli* for bactericidal antibiotics operating through the generation of hydroxyl radicals, regardless of their specific cell targets, based on te fact that HPF fluorescence was quenched *in vivo* in cells exposed to bactericidal but not to bacteriostatic compounds (Kohanski *et al.*, 2007). This interpretation was recently refuted by Liu and Imlay (2013) and Keren *et al.* (2013) who found that bactericidal antibiotics kill *E. coli* in the absence of oxygen. In agreement with these reports, Mahoney and Silhavy (2013) found that active oxygen forms are not produced by *E. coli* in response to all bactericidal compounds.

Our results indicated that in *Pseudomonas putida* the pattern of HPF fluorescence quenching by bactericidal and bacteriostatic compounds was similar to that described by Kohanski *et al.* (2007) for *E. coli*. We opted to determine the hydrogen peroxide production, a precursor of the $OH^{\bullet}$ radicals, in *P. putida* DOT-T1E and its isogenic *ttgABC* mutant. In this series of assays we cultured *P. putida* DOT-T1E and DOT-T1E-18 in LB medium and when the cells were in the early exponential phase, different concentrations of ampicillin or chloramphenicol were added so that 0.5x and 1x MIC concentrations were reached. The results obtained showed that the levels of hydrogen peroxide did not increase over a low basal level in either the wild-type strain or the mutant in response to the different doses of these antibiotics.

In order to study if in response to ampicillin the cells activate the oxidative stress response programme we carried out transcriptomic assays and analysed the expression

of a number of oxidative stress genes using quantitative Real Time-PCR (qRT-PCR). To this end total RNA was extracted from DOT-T1E cells exposed to or not exposed to 300 µg/ml ampicillin and global expression analyses performed. We observed that in response to ampicillin 57 genes increased their expression and 22 genes were repressed (see Table 3, Suppl. text and Suppl. Table 2); however, we found no genes related to oxidative stress were regulated in the presence of ampicillin. In contrast we found that genes related to general stress such as *recA* and *lexA* genes were induced. To further confirm the array assays we carried out qRT-PCR assays to quantify the level of induction of genes encoding alkyl hydroperoxidase (T1E_5238), catalases (T1E_3279 and T1E_4765) and catalase peroxidase (T1E_1753) and the *recA* gene. We found that while oxidative stress genes were not induced in cells growing in the presence of ampicillin (relative expression level was 0.88-1.4), the expression of *recA* increased by 6-fold. In *P. putida* DOT-T1E18, which exhibited enhanced sensitivity to ampicillin, no induction of oxidative stress genes was observed either in response to ampicillin (Suppl. Material and Suppl. Tables 3 and 4). This set of results suggests that ampicillin does not lead to the generation of reactive oxygen species (ROS) and that no oxygen stress genes are induced in response to ampicillin, while genes related to the general stress response were induced.

Indole is an interspecies signaling molecule.

Genome analysis of DOT-T1E did not reveal the presence of tryptophanase orthologs in this strain (Udaondo *et al.*, 2013) and to test whether or not indole was produced by DOT-T1E through another pathway, we examined culture supernatants from *P. putida* DOT-T1E grown on minimal medium with and without tryptophan. No indication of indole production by *P. putida* was found in the HPLC-MS analysis. In a

series of parallel control assays with *E. coli* LK111 (Table 1) we found, in accordance to Lee *et al.* (2007), that *E. coli* produced indole at concentrations up to 400 µM when cells reached the early stationary phase.

Since indole has been described as an inter-species signalling molecule, we decided to examine whether co-culturing of *E. coli* with *P. putida* DOT-T1E and its mutants had an effect on antibiotic resistance in this soil bacterium. To this end, first we cultured *E. coli* LK111 and its isogenic *tnaA* (tryptophanase) mutant in LB with tryptophan and the filtered culture supernatant was used as a source of indole to test for antibiotic resistance in DOT-T1E, T1E-18 and T1E-PS28 using the inhibition halo test (Figure 2). We found that the parental strain (Figure 2A) and T1E-PS28 (Figures 2A and 2C) exhibited a similar level of resistance to antibiotics in the presence and in absence the of *E. coli* culture supernatants. The inhibition halo of T1E-18 in the absence of the culture supernatant or with the supernatant from the *tnaA* mutant was larger than that of the parental strain and this difference was particularly notable with ticarcillin, ampicillin and chloramphenicol (Figure 2). Meanwhile, the addition of *E. coli* LK111 supernatants enhanced tolerance to all antibiotics reaching resistance levels as high as with pure indole. This indicates that indole produced by enteric bacteria was effective in promoting antibiotic resistance in *P. putida*. This was further confirmed by monitoring *P. putida* growth with time. In fact, Figure 3 shows that while 200 µg/ml of ampicillin inhibited the growth of DOT-T1E-18, the addition of *E. coli* LK111 culture supernatant it was sufficient to alleviate this effect; although a short delay was observed before the cells entered log phase and growth rates 20% slower than in the absence of antibiotics.

We subsequently tested the *in vivo* growth of *P. putida* wild type and DOT1E-18 and indole producing *E. coli* strains in the presence and in the absence of antibiotics. To this end *E. coli* and each of the *P. putida* strains were inoculated at equivalent cell

densities (10^5 CFU/ml) and growth was carried out in LB without antibiotics or with ampicillin. As controls, each of the strains were inoculated alone. The number of *E. coli* cells in the co-culture was determined by plating cells on LB + ampicillin + streptomycin - a selective medium for the *E. coli* strain - whereas LB + rifampicin was used for DOT-T1E and LB + rifampicin + kanamycin for T1E-18. We found that after 8 h the co-culture had reached the stationary phase. In the control culture and in each of the co-cultures the *E. coli* cell density was around 10^8 CFU/ml and similar numbers were obtained for T1E or T1E-18. This indicated that in the absence of antibiotics the strains were equally fit. We repeated the above co-culture assays but in the presence of 300 µg/ml ampicillin, *E. coli* is resistant to this drug because it carries the *bla* gene on plasmid pMRS101, the DOT-T1E strain is also resistant to this drug because it is mainly effluxed by the TtgABC pump, and T1E-18 is a strain whose growth is restrained by ampicillin (See Figure 4). Co-cultures of *E. coli* and DOT-T1E cells and of *E. coli* and T1E-18 were prepared with 10^5 CFU/ml of each strain. We found that T1E cell density after 8 h was in the range of 10^8 CFU/ml regardless of whether the *E. coli* strain was present in the culture medium or not, but T1E-18 was able to grow in the presence of ampicillin if and only if *E. coli* LK111 was present in the medium (Figure 4). This indicated that the interspecies signalling is functional and that *Pseudomonas putida* proliferates in media with antibiotics due to the presence of a chemical signal produced by *E. coli*.

Indole produces a wide range of transcriptional responses in DOT-T1E.

Since the above series of results supported that indole is a potential secondary signal molecule in intra-species and inter-species communication, we decided to examine global transcriptomic responses to indole in *P. putida* DOT-T1E and T1E-18.

The assays were carried out with cells growing exponentially in LB medium. Cultures were divided in two halves and 300 μM indole was added to one of them. This concentration was chosen because it is equivalent to that produced by *E. coli* and because we have found that this concentration does not have any detrimental effect on *P. putida* cell growth rates. We found that DOT-T1E induced 43 genes and repressed 23 genes (Table 4 and Suppl. Table 5) in response to indole. As expected from *in vitro* assay results, we found that the *ttgV* and the *ttgGHI* operon were induced in response to indole, but no the *ttgABC* operon. Cells also induced a number of genes involved in energy generation such as the Entner-Doudoroff pathway for glucose metabolism (T1E_1987, T1E_1988, T1E_2001) and the pyruvate dehydrogenase component (T1E_2648), which is likely related to the need to increase feeding of the TCA cycle and to keep it under appropriate operational conditions. A number of iron transport systems were also induced (T1E_1068, T1E_2509, T1E_5142); a response which may be related to the need to build cytochromes for the Nuo respiratory chain. Some enzymes related to oxygen stress such, an alkylhydroperoxide dehydrogenase (T1E_5239), were also induced suggesting that indole gives rise to a soft oxidative stress response. In agreement with this light stress is that only one chaperone, DnaK (T1E_0654), was induced more than 3-fold in response to indole. A number of regulatory proteins whose targets are unknown were also induced (Table 4); it is possible that induction of some of the previously mentioned genes is under the direct/indirect control of these regulatory proteins. A number of genes encoding proteins involved in the glutamine/glutamate cycle were also induced, as well as enzymes related to amino acid metabolism. To further confirm these results we carried out qRT-PCR assays with 5 genes that encoded 6-phosphogluconate dehydratase (T1E_1987, *edd*); an aldehyde dehydrogenase (T1E_4523), the *dnaK* gene (T1E_0654),

an outer membrane ferric siderophore receptor (T1E_5142), a hypothetical protein (T1E_0256) and TtgV; and we found that all were induced 3.8- to 6.3-fold with respect to the levels in the absence of indole.

Equivalent assays to those described above were also carried out with T1E-18 (*ttgABC*). We observed that in the T1E-18 strain the efflux pump *ttgGHI* was induced 2.6 to 3.7-fold (Table 5). We also found that in T1E-18 additional genes not found to be induced in the parental strain were in fact induced; specifically these were genes involved in metabolism of branched amino acids (T1E_3322 through T1E_3325, T1E_5100 and T1E_5101), as well as other genes related to amino acid metabolism. This series of results show that the deficiency in TtgABC and the induction of TtgGHI influence the response of *P. putida* to indole.

DISCUSSION

Bactericidal and bacteriostatic antibiotics are extruded through the TtgABC/TtgGHI RND efflux pumps in *Pseudomonas putida*.

In *Pseudomonas putida* the main mechanism of resistance to bactericidal and bacteriostatic antibiotics is their extrusion by the TtgABC pump (Godoy *et al.*, 2010; Fernández *et al.*, 2012). Our results indicate that bacteriostatic compounds such as chloramphenicol, erythromycin and tetracycline hamper cell growth while 100% of the cells remain viable; whereas ampicillin and norfloxacin, two bactericidal compounds, first inhibit cell growth and then lead to cell death.

The role of TtgABC in bacteriostatic and bactericidal compound extrusion is clear because the T1E-18 mutant exhibited increased antibiotic sensitivity to the tested

antibiotics compared to the wild-type strain (See Table 2). Another efflux pump, TtgGHI, was shown to act as a secondary efflux pump in antibiotic resistance; as demonstrated by the fact that the double *ttgABC*/*ttgGHI* mutant was more sensitive to these drugs than both the parental and the single mutants (see Table 2).

We found that neither bacteriostatic nor bactericidal compounds supplied at different concentrations led to production of hydrogen peroxide. In agreement with this observation is the fact that in the presence of ampicillin or chloramphenicol no induction of oxygen stress genes was observed under our experimental conditions or in other studies (Fernández *et al.*, 2012). Therefore, this set of results are in line with the recent reports that refute that bactericidal compounds act through a common mechanism involving the production of ROS (Keren *et al.*, 2013; Liu and Imlay, 2013; Mahoney and Silhavy, 2013).

Nonetheless, it is interesting to note that the original proposal of a common mechanism for bactericidal killing by Kohanski *et al.* (2007) was based on the inhibition of HPF autofluorescence in the presence of bactericidal compounds, a phenomenon that did not occur in the presence of bacteriostatic compounds. We tested whether quenching of HPF fluorescence occurred in response to different antibiotics in *Pseudomonas putida* DOT-T1E. In accordance with Kohanski *et al.* (2007) we found that in the *P. putida* DOT-T1E strain certain bactericidal antibiotics provoked the inhibition of HPF autofluorescence, which was indeed not the case with bacteriostatic antibiotics such as chloramphenicol and tetracycline (See Figure 1). That quenching of HPF is related to the presence of bactericidal compounds is clear because in the T1E-18 mutant, devoid of the main antibiotic efflux pump, HPF fluorescence quenching occurred at lower ampicillin concentrations than in the parental strain and at even lower concentrations in the double mutant devoid of both TttgABC and TtgGHI activity.

Concomitantly for T1E-18 in the presence of indole, resistance to bactericidal compounds increased and HPF quenching in response to these compounds was partially alleviated; these results indicate a clear correlation between the two phenotypes, although the molecular basis of these observations are unknown.

Indole is a signal molecule recognized by *P. putida*

In enterobacteria indole behaves as an intra-species signalling molecule because it increases resistance to bactericidal and bacteriostatic antibiotics. Indole is also an inter-species signalling molecule because it controls the phenotypes of eukaryotic and prokaryotic cells that are unable to produce it. It has also been described that indole producing commensal *E. coli* strains influence gene expression in human epithelial cells leading to tighter cell-junctions while increasing the beneficial effect of cytokines (Bansal *et al.*, 2010) and inhibiting the colonization of enterohaemorrhagic *E. coli* strains in gastrointestinal tracts (Bansal *et al.*, 2007). Therefore, indole could even be considered and inter-kingdom signal.

A characteristic of a molecule to be considered as a signalling molecule is that it should influence the expression of a range of genes. Indeed global transcriptional studies with a number of Gram-negative bacteria have shown that signalling molecules involved in cell-cell communication, such as *N*-acyl homoserine lactone, cyclic peptides, and quinolones ((Fuqua *et al.*, 1994; Salmond *et al.*, 1995; Fuqua *et al.*, 1996; Holden *et al.*, 1999; Pesci *et al.*, 1999) influence the pattern of expression of microbes perceiving the signal. In this study we report that indole acts as an extracellular signaling molecule capable of altering the pattern of expression of 66 genes in the wild-type *P. putida* DOT-T1E (43 induced and 23 repressed, Tables 4 and Suppl. Table 5). Indole not only induces antibiotic efflux pumps that endow antibiotic-sensitive strains

with competitive advantages to thrive in a culture medium containing antibiotics, but also a range of genes associated with 8 COG groups, namely, amino acid metabolism, inorganic ion metabolism, efflux pumps, energy production and carbohydrate metabolism, metal transport systems, oxygen stress, regulatory proteins and hypothetical proteins. The results presented in this study suggest that the TtgGHI pump is part of a circuit related to bacterial cell communication because it is induced by a signalling molecule produced by other microorganisms; through its induction the *P. putida* strain is able to thrive under adverse conditions. In *Pseudomonas* indole induced catabolism of amino acids, such as arginine, glutamine and glutamate. This is in agreement with the observations in *E. coli* by Lee *et al.* (2007) and Wang *et al.* (2001) and with the studies by Zinser and Kolter (1999) showing that the ability to catabolize amino acids is an important parameter to persist and compete in the stationary phase. This finding points towards the possibility of indole signalling playing a role in a pathway that prepares the cells for a nutrient-poor environment in which the catabolism of amino acids becomes important for energy production.

In summary our results support that in *Pseudomonas putida* the RND efflux pumps are relevant for antibiotic resistance and that these pumps can be induced by substrates, as is the case for TtgABC, or by signal molecules, such as indole, in the case of the TtgGHI efflux pump. Pumps that are induced by substrates can be regarded as self-autonomous elements of the microorganism in regard to antibiotic resistance, while those induced by signalling, particularly inter-species signalling can be considered part of the programme triggered by bacterial communities. This is relevant in the context of infections and antibiotic treatments. As we know antibiotics have been used extensively to treat infections, but along with their use comes the problem of increasing microbial resistance, which results in a dramatic drop in the effectiveness of the therapies. The

identification of signalling molecules responsible for the induction of antibiotic resistance is therefore considered very important to the fight against antibiotic resistance, and is undoubtedly of vital importance for infections caused by multiple microbes.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Bacterial cells were grown in liquid Luria-Bertani (LB) medium at 30 °C with shaking on an orbital platform operating at 200 rpm (Kühner incubator). When necessary the appropriate antibiotics were added to reach the following final concentrations: 50 µg/ml kanamycin and 20 µg/ml rifampicin. The concentrations of other antibiotics used in this study are indicated in the text.

Co-culture assays of E. coli *and* P. putida *in the presence of ampicillin*

For this series of assays different cells were inoculated into 20 ml LB and grown individually for 12-14 h in the absence of antibiotics. The turbidity of these cultures was adjusted to an optical density at 660 nm of 0.1 ($1\pm0.1\times10^5$ CFU/ml) with sterile LB broth, and then 10 ml of *E. coli* cells was mixed with 10 ml of *P. putida* DOT-T1E or *P. putida* T1E-18. Pure culture experiments for each strain were also conducted as controls. When indicated ampicillin (300 µg/ml) was added to all cultures and subsequently incubated for 20 h with agitation at 30 °C. Samples were removed at the end of the assay and serial dilutions plated on selective media. Solid LB medium supplemented with 20 µg/ml rifampicin was used to enumerate *P. putida* DOT-T1E; solid LB with rifampicin and kanamycin was used to count *P. putida* T1E-18, and LB supplemented with 50 µg/ml of streptomycin and 100 µg/ml of ampicillin was used to

count *E. coli* K111. After 24 h at 30 ºC colonies were counted and CFU/ml were inferred. Each experiment was repeated at least three times.

Minimum inhibitory concentration (MIC)

MIC assays were performed in liquid LB medium in the presence or absence of 300 µM of indole using the two-fold serial dilution test according to the guidelines of the Clinical and Laboratory Standards Institute (2003). The highest concentration of the antibiotics used were: tetracycline (10,000 µg/ml); chloramphenicol (3,000 µg/ml); norfloxacin (200 µg/ml); erythromycin (3,000 µg/ml); and ampicillin (10,000 µg/ml). At least three independent experiments were carried out for each determination and each experiment was run in triplicate. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of antibiotic that inhibited the growth of the strain by >90%.

Disk diffusion antibiotic susceptibility testing

The Kirby–Bauer technique was used (Bauer *et al.*, 1966). Briefly, Luria-Bertani agar plates with or without indole (300 µM) were spread with a suspension of approximately 10^8 CFU/ml of wild-type *P. putida* DOT-T1E or its mutant strains (*P. putida* T1E-18, T1E-PS28 and T1E-PS32) to produce a lawn. Once the plate surface was dried, antibiotic disks of ofloxacin (5 µg); pefloxacin (5 µg); amoxicillin (25 µg); ticarcillin (75 µg); ampicillin (10 µg); ceftazidime (30 µg); chloramphenicol (30 µg); erythromycin (15 µg); and tetracycline (30 µg) (BioMerieux, Spain) were placed on the surface of plates. After 18-20 h at 30 ºC, the inhibition zone (in millimeters) was measured around each disk. For the series of assays in which *E. coli* supernatants were used as a source of indole, 100 ml of the filtered culture supernatants were mixed with

50 ml of 5% LB agar and spread on plates; the assays were done as described above when pure indole was used.

DNA microarrays.

A *Pseudomonas putida* array (Progenika, Spain) was used for transcriptomic studies; the array contains 5539 gene-specific oligonucleotides (50-mer) spotted in duplicate onto γ-amino silane-treated 25 × 75 microscope slides and bound to the slide with UV light and heat (Yuste *et al.*, 2006). *Pseudomonas putida* DOT-T1E, T1E-18 and T1E-PS28 were grown overnight in LB medium and used to inoculate fresh medium with or without indole at a concentration of 300 μM. Cultures were incubated at 30 ºC until a turbidity of 0.5-0.6 at 660 nm (exponential phase) was reached. Cells were then harvested and immediately subjected to RNA extraction. RNA was isolated using the TRI-reagent/BCP method and the subsequent preparation of fluorescently-labelled cDNA (Yuste *et al.*, 2006; Duque *et al.*, 2007). Hybridization conditions and data collection were carried out as previously described (Yuste *et al.*, 2006). Data were normalized by applying the LOWESS intensity-dependent normalization method (Yang *et al.*, 2002) and statistically analyzed with the Almazen System software (Alma Bioinformatics S.L, Spain.). P-values were calculated with the Student's *t* test. Genes were considered differentially expressed when the fold change was at least 2 and the P-value was ≤ 0.05. Microarray data were deposited in the Array Express Archive database (www.ebi.ac.uk/arrayexpress) under accession numbers E-MEXP-3819, 3822, 3823 and 3824.

HPF fluorescence experiments using flow cytometry

We used the fluorescent reporter dye 3'-(*p*-hydroxyphenyl) fluorescein (HPF, Invitrogen) at a concentration of 5 µM and the following PMT voltage settings were used: E00 (FSC), 360 (SSC) and 825 (FL1). Flow data were processed and analyzed with FACSDiva 6.0. In all experiments cells were grown overnight and then diluted 1:1000 in 25 ml LB supplemented with 5 µM HPF. Flasks were incubated in a light-insulated shaker at 30 °C and 200 rpm. Antibiotics were added (see details in the Results section) at the early exponential phase, and samples were taken immediately before the addition of the drug (time zero) and 3 h later. At these time points, approximately 10^7 cells were collected, washed once, and resuspended in filtered PBS (pH 7.2) prior to measurement of fluorescence.

Bioscreen assays

Freshly grown individual colonies of *P. putida* DOT-T1E and DOT-T1E-18 strains from LB plates supplemented with 10 µg/ml rifampicin were picked and grown overnight in liquid LB medium at 30 ºC. The cultures were resuspended in 15 ml of liquid LB medium to an optical density of 0.1 at 660 nm. The wells of the microplates were filled with 190 µl of liquid LB medium or 170 µl of *E. coli* LK111 filtered supernatant, both supplemented with ampicillin (200 µg/ml), and 10 µl of resuspended culture. In the case of the *E. coli* supernatant, 20 µl of liquid 10xLB medium was added. Positive control wells consisted of liquid LB medium inoculated with DOT-T1E strains, and negative control wells contained medium without cells. Growth was monitored using a type FP-1100-C Bioscreen C MBR analyzer system (OY Growth Curves Ab Ltd., Raisio, Finland) at 30 ºC with continuous agitation. Turbidity was measured using a sideband filter at 420 to 580 nm every 60 min over a 24 h period. Each strain was

assayed at least three times for each of the compounds tested, and plates were visually examined following each assay to verify the results (Daniels *et al.*, 2010).

Quantitative real-time PCR

The extraction of RNA was performed as previously described. The primers used for real-time PCR analyses are listed in Suppl. Table 7. Real-time PCR amplification was carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical system software (version 2.1.97.1001). Each 25-µl reaction mixture contained 12.5 µl iQ SYBR green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 µM each dNTP, iTaq DNA polymerase [50 U ml^{-1}], 6 mM $MgCl_2$, SYBR green I, 20 nM fluorescein, and stabilizers [Bio-Rad] [0.4 µM for each primer]) and 2 µl template cDNA (diluted 10- or 1,000-fold). Thermal cycling conditions were: one cycle at 95°C for 10 min and then 40 cycles at 95°C for 15 s, 61.1°C or 61.7ºC (for indole and ampicillin experiments respectively) for 30 s, and 72°C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. A final extension cycle (72°C for 1 min) was performed. The PCR products were between 132 and 350 bp. Melting curve analysis was performed by gradually heating the PCR mixture from 55 to 95°C at a rate of 0.5°C per 10 s for 80 cycles. The relative expression of the genes was normalized to that of 16S rRNA, and the results were analyzed by means of the comparative cycle threshold ($\Delta\Delta C_T$) method (Pfaffl, 2001).

Measurement of H_2O_2 production

The rate of H_2O_2 formation by antibiotic-treated cells was measured with *P. putida* DOT-T1E strain. Cultures of DOT-T1E cells were grown overnight at 30°C and

diluted to a turbidity of 0.01. Ampicillin (300 μg/ml) was added at early-exponential phase. At various time points cells were centrifuged and the pellets were resuspended, washed once in 1xM9 buffer, and resuspended again in 1 ml of lysis buffer containing Tris 50 mM, pH 7.5, 50 mM NaCl, 2 mM EDTA, 4 mM β-mercaptoethanol, and 1x Complete™ protease inhibitor mixture (Roche Applied Science). Cells were lysed by sonication and the insoluble fraction was separated and discarded follow centrifugation at 18000 × *g* for 15 min (Domínguez-Cuevas *et al.*, 2006). H_2O_2 production was measured in a spectrophotometer at 492 nm as described by Buege and Aust (1978) using a reactive solution made of trichloroacetic (10%), ferrous ammonium sulfate (70 mM) and potassium thiocyanate (2.5 M).

ACKNOWLEDGEMENTS

This work was supported by FEDER Grants through the Junta de Andalucía programme of excellence and Plan Nacional.

REFERENCES

Anyanful, A., Dolan-Livengood, J.M., Lewis, T., Sheth, S., Dezalia, M.N., Sherman, M.A. *et al.* (2005) Paralysis and killing of Caenorhabditis elegans by enteropathogenic Escherichia coli requires the bacterial tryptophanase gene. *Mol Microbiol* **57**: 988-1007.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M. *et al.* (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006 0008.

Bansal, T., Alaniz, R.C., Wood, T.K., and Jayaraman, A. (2010) The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U S A* **107**: 228-233.

Bansal, T., Englert, D., Lee, J., Hegde, M., Wood, T.K., and Jayaraman, A. (2007) Differential effects of epinephrine, norepinephrine, and indole on Escherichia coli O157:H7 chemotaxis, colonization, and gene expression. *Infect Immun* **75**: 4597-4607.

Bauer, A.W., Kirby, W.M., Sherris, J.C., and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**: 493-496.

Buege, J.A., and Aust, S.D. (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302-310.

Chant, E.L., and Summers, D.K. (2007) Indole signalling contributes to the stable maintenance of Escherichia coli multicopy plasmids. *Mol Microbiol* **63**: 35-43.

Chu, W., Zere, T.R., Weber, M.M., Wood, T.K., Whiteley, M., Hidalgo-Romano, B. *et al.* (2012) Indole production promotes Escherichia coli mixed-culture growth with Pseudomonas aeruginosa by inhibiting quorum signaling. *Appl Environ Microbiol* **78**: 411-419.

Daniels, C., and Ramos, J.L. (2009) Adaptive drug resistance mediated by root-nodulation-cell division efflux pumps. *Clin Microbiol Infect* **15 Suppl 1**: 32-36.

Daniels, C., Godoy, P., Duque, E., Molina-Henares, M.A., de la Torre, J., Del Arco, J.M. *et al.* (2010) Global regulation of food supply by Pseudomonas putida DOT-T1E. *J Bacteriol* **192**: 2169-2181.

Domínguez-Cuevas, P., Gonzalez-Pastor, J.E., Marques, S., Ramos, J.L., and de Lorenzo, V. (2006) Transcriptional tradeoff between metabolic and stress-response programs in Pseudomonas putida KT2440 cells exposed to toluene. *J Biol Chem* **281**: 11981-11991.

Duque, E., Segura, A., Mosqueda, G., and Ramos, J.L. (2001) Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of Pseudomonas putida. *Mol Microbiol* **39**: 1100-1106.

Duque, E., Rodriguez-Herva, J.J., de la Torre, J., Dominguez-Cuevas, P., Munoz-Rojas, J., and Ramos, J.L. (2007) The RpoT regulon of Pseudomonas putida DOT-T1E and its role in stress endurance against solvents. *J Bacteriol* **189**: 207-219.

Fernández, M., Conde, S., de la Torre, J., Molina-Santiago, C., Ramos, J.L., and Duque, E. (2012) Mechanisms of resistance to chloramphenicol in Pseudomonas putida KT2440. *Antimicrob Agents Chemother* **56**: 1001-1009.

Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50**: 727-751.

Fuqua, S.A., Oesterreich, S., Hilsenbeck, S.G., Von Hoff, D.D., Eckardt, J., and Osborne, C.K. (1994) Heat shock proteins and drug resistance. *Breast Cancer Res Treat* **32**: 67-71.

Godoy, P., Molina-Henares, A.J., de la Torre, J., Duque, E., and Ramos, J.L. (2010) Characterization of the RND family of multidrug efflux pumps: in silico to in vivo confirmation of four functionally distinct subgroups. *Microb Biotechnol* **3**: 691-700.

Han, T.H., Lee, J.H., Cho, M.H., Wood, T.K., and Lee, J. (2011) Environmental factors affecting indole production in Escherichia coli. *Res Microbiol* **162**: 108-116.

Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T., and Yamaguchi, A. (2005) Indole induces the expression of multidrug exporter genes in Escherichia coli. *Mol Microbiol* **55**: 1113-1126.

Hirakawa, H., Kodama, T., Takumi-Kobayashi, A., Honda, T., and Yamaguchi, A. (2009) Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic Escherichia coli O157:H7. *Microbiology* **155**: 541-550.

Holden, M.T., Ram Chhabra, S., de Nys, R., Stead, P., Bainton, N.J., Hill, P.J. *et al.* (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from Pseudomonas aeruginosa and other gram-negative bacteria. *Mol Microbiol* **33**: 1254-1266.

Jakovleva, J., Teppo, A., Velts, A., Saumaa, S., Moor, H., Kivisaar, M., and Teras, R. (2012) Fis regulates the competitiveness of Pseudomonas putida on barley roots by inducing biofilm formation. *Microbiology* **158**: 708-720.

Kamath, A.V., and Vaidyanathan, C.S. (1990) New pathway for the biodegradation of indole in Aspergillus niger. *Appl Environ Microbiol* **56**: 275-280.

Keren, I., Wu, Y., Inocencio, J., Mulcahy, L.R., and Lewis, K. (2013) Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* **339**: 1213-1216.

Kim, D., Sitepu, I.R., and Hashidoko, Y. (2013) Induction of biofilm formation in the betaproteobacterium Burkholderia unamae CK43B exposed to exogenous indole and gallic acid. *Appl Environ Microbiol* **79**: 4845-4852.

Kobayashi, A., Hirakawa, H., Hirata, T., Nishino, K., and Yamaguchi, A. (2006) Growth phase-dependent expression of drug exporters in Escherichia coli and its contribution to drug tolerance. *J Bacteriol* **188**: 5693-5703.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797-810.

Lee, J., Jayaraman, A., and Wood, T.K. (2007) Indole is an inter-species biofilm signal mediated by SdiA. *BMC Microbiol* **7**: 42.

Lee, J., Attila, C., Cirillo, S.L., Cirillo, J.D., and Wood, T.K. (2009) Indole and 7-hydroxyindole diminish Pseudomonas aeruginosa virulence. *Microb Biotechnol* **2**: 75-90.

Lee, J.H., and Lee, J. (2010) Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev* **34**: 426-444.

Liu, Y., and Imlay, J.A. (2013) Cell death from antibiotics without the involvement of reactive oxygen species. *Science* **339**: 1210-1213.

Mahoney, T.F., and Silhavy, T.J. (2013) The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. *J Bacteriol* **195**: 1869-1874.

March, J.C., and Bentley, W.E. (2004) Quorum sensing and bacterial cross-talk in biotechnology. *Curr Opin Biotechnol* **15**: 495-502.

Martino, P.D., Fursy, R., Bret, L., Sundararaju, B., and Phillips, R.S. (2003) Indole can act as an extracellular signal to regulate biofilm formation of Escherichia coli and other indole-producing bacteria. *Can J Microbiol* **49**: 443-449.

Molina-Henares, A.J., Krell, T., Eugenia Guazzaroni, M., Segura, A., and Ramos, J.L. (2006) Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. *FEMS Microbiol Rev* **30**: 157-186.

Mosqueda, G., and Ramos, J.L. (2000) A set of genes encoding a second toluene efflux system in Pseudomonas putida DOT-T1E is linked to the tod genes for toluene metabolism. *J Bacteriol* **182**: 937-943.

Nikaido, E., Yamaguchi, A., and Nishino, K. (2008) AcrAB multidrug efflux pump regulation in Salmonella enterica serovar Typhimurium by RamA in response to environmental signals. *J Biol Chem* **283**: 24245-24253.

Pesci, E.C., Milbank, J.B., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., and Iglewski, B.H. (1999) Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A* **96**: 11229-11234.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Ramos, J.L., Duque, E., Huertas, M.J., and Haidour, A. (1995) Isolation and expansion of the catabolic potential of a Pseudomonas putida strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J Bacteriol* **177**: 3911-3916.

Ramos, J.L., Duque, E., Godoy, P., and Segura, A. (1998) Efflux pumps involved in toluene tolerance in Pseudomonas putida DOT-T1E. *J Bacteriol* **180**: 3323-3329.

Ramos, J.L., Martinez-Bueno, M., Molina-Henares, A.J., Teran, W., Watanabe, K., Zhang, X. *et al.* (2005) The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* **69**: 326-356.

Roca, A., Rodriguez-Herva, J.J., Duque, E., and Ramos, J.L. (2008) Physiological responses of Pseudomonas putida to formaldehyde during detoxification. *Microb Biotechnol* **1**: 158-169.

Rodríguez-Herva, J.J., Duque, E., Molina-Henares, M.A., Navarro-Aviles, G., Van Dillewijn, P., De La Torre, J. *et al.* (2010) Physiological and transcriptomic characterization of a fliA mutant of Pseudomonas putida KT2440. *Environ Microbiol Rep* **2**: 373-380.

Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J.L., and Segura, A. (2001) Three efflux pumps are required to provide efficient tolerance to toluene in Pseudomonas putida DOT-T1E. *J Bacteriol* **183**: 3967-3973.

Salmond, G.P., Bycroft, B.W., Stewart, G.S., and Williams, P. (1995) The bacterial 'enigma': cracking the code of cell-cell communication. *Mol Microbiol* **16**: 615-624.

Sarker, M.R., and Cornelis, G.R. (1997) An improved version of suicide vector pKNG101 for gene replacement in gram-negative bacteria. *Mol Microbiol* **23**: 410-411.

Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H.J., and Nagano, T. (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J Biol Chem* **278**: 3170-3175.

Terán, W., Felipe, A., Segura, A., Rojas, A., Ramos, J.L., and Gallegos, M.T. (2003) Antibiotic-dependent induction of Pseudomonas putida DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. *Antimicrob Agents Chemother* **47**: 3067-3072.

Terán, W., Felipe, A., Fillet, S., Guazzaroni, M.E., Krell, T., Ruiz, R. *et al.* (2007) Complexity in efflux pump control: cross-regulation by the paralogues TtgV and TtgT. *Mol Microbiol* **66**: 1416-1428.

Udaondo, Z., Molina, L., Daniels, C., Gomez, M.J., Molina-Henares, M.A., Matilla, M.A. *et al.* (2013) Metabolic potential of the organic-solvent tolerant Pseudomonas putida DOT-T1E deduced from its annotated genome. *Microb Biotechnol* **6**: 598-611.

Udaondo, Z., Duque, E., Fernandez, M., Molina, L., de la Torre, J., Bernal, P. *et al.* (2012) Analysis of solvent tolerance in Pseudomonas putida DOT-T1E based on its genome sequence and a collection of mutants. *FEBS Lett* **586**: 2932-2938.

Ueda, A., and Wood, T.K. (2009) Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in Pseudomonas aeruginosa through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog* **5**: e1000483.

Vega, N.M., Allison, K.R., Samuels, A.N., Klempner, M.S., and Collins, J.J. (2013) Salmonella typhimurium intercepts Escherichia coli signaling to enhance antibiotic tolerance. *Proc Natl Acad Sci U S A* **110**: 14420-14425.

Vert, G., and Chory, J. (2011) Crosstalk in cellular signaling: background noise or the real thing? *Dev Cell* **21**: 985-991.

Wang, D., Ding, X., and Rather, P.N. (2001) Indole can act as an extracellular signal in Escherichia coli. *J Bacteriol* **183**: 4210-4216.

Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* **30**: e15.

Yanofsky, C., Horn, V., and Gollnick, P. (1991) Physiological studies of tryptophan transport and tryptophanase operon induction in Escherichia coli. *J Bacteriol* **173**: 6009-6017.

Yousef-Coronado, F., Travieso, M.L., and Espinosa-Urgel, M. (2008) Different, overlapping mechanisms for colonization of abiotic and plant surfaces by Pseudomonas putida. *FEMS Microbiol Lett* **288**: 118-124.

Yuste, L., Hervas, A.B., Canosa, I., Tobes, R., Jimenez, J.I., Nogales, J. *et al.* (2006) Growth phase-dependent expression of the Pseudomonas putida KT2440 transcriptional machinery analysed with a genome-wide DNA microarray. *Environ Microbiol* **8**: 165-177.

Zinser, E.R., and Kolter, R. (1999) Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. *J Bacteriol* **181**: 5800-5807.

LEGEND FOR FIGURES

Figure 1. HPF quenching in *P. putida* DOT-T1E and mutants in the presence of indole and antibiotics. (A, D, G and J) Log change in colony-forming units per milliliter (CFU/ml) by *P. putida* DOT-T1E, T1E-PS28, T1E-18 and T1E-PS32 respectively. Symbols: the no-drug control is represented by grey triangles; Black circles, culture with ampicillin added; orange circles, cultures with simultaneous addition of ampicillin and indole; purple squares, cultures with chloramphenicol; green squares, cultures with chloramphenicol and indole. Error bars represent ±SD of the mean.

(B, E, H and K) HPF quenching 3 h after the addition of the drug chloramphenicol to *P. putida* DOT-T1E (150 µg/ml), T1E-PS28 (150 µg/ml), T1E-18 (70 µg/ml) and T1E-PS32 (2 µg/ml) cultures. Symbols: the baseline is represented by green line; grey line, baseline; red line, chloramphenicol; and blue line, indole (300 µM) and chloramphenicol.

(C, F, I and L) HPF quenching 3 h after the addition of the drug ampicillin to *P. putida* DOT-T1E (300 µg/ml), T1E-PS28 (300 µg/ml), T1E-18 (100 µg/ml) and T1E-PS32 (20 µg/ml) cultures. Symbols: the baseline is represented by green line; grey line, baseline; red line, ampicillin; and blue line, indole (300 µM) and ampicillin.

Figure 2. Effect of *E. coli* K12 LK111 supernatant on antibiotic resistance in the *P. putida* DOT-T1E, T1E-18 and T1E-PS28 strains. Inhibition halos (in mm) produced by several antibiotics. From left to right and top to bottom: ofloxacin, pefloxacin, amoxicillin, ticarcillin, ampicillin, ceftazidime, chloramphenicol and tetracycline in the

presence (black) and in the absence (open) of 300 µM indole. (A), *P. putida* DOT-T1E; (B), *P. putida* T1E-PS28; and (C), *P. putida* T1E-18.

Figure 3. *E. coli* supernatant effect on the growth of *P. putida* DOT-T1E, T1E-18 and T1E-PS28 strains in the presence and absence of ampicillin. *P. putida* DOT-T1E, T1E-18 and T1E-PS28 were grown overnight at 30 ºC in LB liquid medium. The cultures were diluted in LB medium or spent-LB medium in which *E. coli* K111 had been grown (this medium contained about 400 µM indole), in the latter case 10×LB was added to reach a 1×LB concentration. Half of the samples were used as a control and to the other 100 µg/ml Ap was added. Growth was followed by measuring the turbidity every hour using a Bioscreen for 24 hours. (A) Wild-type strain; (B) *P. putida* DOT-T1E-18; (C) *P. putida* DOT-T1E-PS28○ control LB; ● LB with ampicillin; Δ *E. coli* supernatant; ▲ *E. coli* supernatant and ampicillin.

Figure 4. Growth of *P. putida* strains in the presence of *E. coli* K-12 LK111 and ampicillin. *P. putida* DOT-T1E and T1E-18 were grown with 300 µg/ml ampicillin in the presence and in the absence of *E. coli* K-12 LK111 and were selected in LB with Rif and in LB with Km. The Figure represents the CFU of *P. putida* DOT-T1E or T1E-18 per ml of culture in presence and absence of *E. coli* K-12.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source of reference
***P. putida* strains**		
DOT-T1E	Rif^r	Ramos *et al.* (1998)
T1E-18	DOT-T1E, Rif^r, Km^r, *ttgB*::'*phoA*-Km	Ramos *et al.* (1998)
T1E-PS28	DOT-T1E, Rif^r, Sm^r, *ttgH*ΩSm	Rojas *et al.* (2001)
T1E-PS32	DOT-T1E, Rif^r, Km^r, Sm^r, *ttgB*::'*phoA*-Km, *ttgH*ΩSm	Rojas *et al.* (2001)
***E. coli* strains**		
K12 LK111	Contains pMRS101, Ap^r, Sm^r	Sarker and Cornelis (1997)
K12 BW25113ΔTnaA	Δ *tnaA*ΩKm^r	Baba *et al.* (2006)

Rif^r, Km^r, Ap^r and Sm^r stand for resistance to rifampicin, kanamycin, ampicillin and streptomycin, respectively

Table 2. Minimum inhibitory concentrations (μ*g* mL^{-1}) of several antibiotics for *P. putida* DOT-T1E

	DOT-T1E		T1E-18		T1E-PS28		T1E-PS32	
Antibiotic (μg/ml)	**-**	**Indole**	**-**	**Indole**	**-**	**Indole**	**-**	**Indole**
Tetracycline	8	8	<1	5	8	8	<1	<1
Ampicillin	625	625	200	300	625	625	40	40
Chloramphenicol	300	300	70	180	300	300	3	3
Norfloxacin	2	2	<1	1	2	2	<1	<1
Erythromycin	400	400	200	300	400	400	100	100

Assay conditions are described under Experimental conditions

Table 3. *Pseudomonas putida* DOT-T1E genes induced in presence to 300 μg/ml ampicillin

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_0851	Oxidoreductase putative	4.2
T1E_1814	Glycine betaine/carnitine/choline ABC transporter permease protein	2.2
T1E_2070	Opine ABC transporter permease protein putative	3.6
T1E_2171	Tryptophan 2-monooxygenase putative	2.2
T1E_2178	Prolyl oligopeptidase family protein	2.8
T1E_2234	Hydrolase haloacid dehalogenase-like family	2.1
T1E_3735	SpeA-biosynthetic arginine decarboxylase	2.0
	Secretion	
T1E_1682	XcpQ-type II secretion pathway protein XcpQ	5.1
T1E_1683	XcpP-type II secretion pathway protein XcpP	2.9
T1E_2177	PqqE-coenzyme PQQ synthesis protein E	2.5
T1E_5608	Clp protease putative	3.1
	DNA metabolism	
T1E_3123	Methyltransferase putative	2.6
T1E_3644	DNA topology modulation kinase FlaR putative	2.7
T1E_4980	*recA*	2.0
	Inorganic ion metabolism	
T1E_0376	Oxidoreductase putative	4.0
T1E_1827	Opine ABC transporter periplasmic binding protein protein	2.0
T1E_2285	Transmembrane sensor putative	2.7

T1E_4628	Dioxygenase TauD/TfdA family	2.1
T1E_5238	Monooxygenase putative	5.3
T1E_5588	PhaM-PhaM protein	2.6
	Energy production and conversion	
T1E_5014	AceA-isocitrate lyase	2.1
T1E_0770	Glyceraldehyde-3-phosphate dehydrogenase NADP-dependent	2.8
	Metal transport systems	
T1E_2504	Outer membrane ferric siderophore receptor putative	2.6
T1E_2591	Iron-sulfur cluster-binding protein putative	2.3
T1E_3391	Outer membrane ferric siderophore receptor	4.0
	Regulatory proteins	
T1E_0578	Transcriptional regulator LysR family	2.0
T1E_0774	Transcriptional regulator AmpR putative	4.3
T1E_0860	Transcriptional regulator Sir2 family	2.3
T1E_0909	Transcriptional regulator TrpI	2.3
T1E_1161	TldD/PmbA family protein	3.0
T1E_2170	Transcriptional regulator AsnC family	2.7
T1E_2269	Transcriptional regulator RpiR family	8.2
T1E_3731	Response regulator	2.2
	Lipid and membrane proteins	
T1E_0012	Periplasmic binding protein putative	2.6
T1E_0548	FadAx-3-ketoacyl-CoA thiolase	2.1
T1E_0917	Lipoprotein putative	3.2
T1E_1598	Membrane protein putative	2.1
T1E_1624	CsgG-curli fiber membrane-associated lipoprotein	2.0
T1E_1888	Lipoprotein putative	2.0

T1E_2185	Acyl-CoA dehydrogenase putative	2.0
T1E_2827	Surface colonization protein putative	3.2
T1E_3742	PbpC-penicillin-binding protein 1C	2.2
T1E_3913	Porin putative	2.7
T1E_3991	Peptidase M23/M37 family	2.7
	putative transmembrane protein-pWW0 79375-79734	2.6
	Hypothetical proteins	
T1E_0905	Conserved hypothetical protein	2.6
T1E_0913	Conserved hypothetical protein	2.0
T1E_0958	Hypothetical protein	3.1
T1E_1593	Conserved hypothetical protein	2.1
T1E_2521	Conserved hypothetical protein	3.0
T1E_2619	Hypothetical protein	4.9
T1E_2712	Conserved hypothetical protein	2.4
T1E_3099	Conserved hypothetical protein	2.0
T1E_3301	Conserved hypothetical protein	2.6
T1E_3302	Hypothetical protein	3.1
T1E_5088	Hypothetical protein	3.0
T1E_5336	Hypothetical protein	3.4

Table 4. *Pseudomonas putida* DOT-T1E genes induced by the presence of 300 µM indole

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_0551	Leucine-rich repeat domain protein	3.4
T1E_1075	Glutamine synthetase putative	2.8
T1E_2070	Opine ABC transporter permease protein putative	5.5
T1E_2506	Glutamate synthase large subunit putative	2.5
T1E_3909	Shikimate 5-dehydrogenase/quinate 5-dehydrogenase family	3.5
T1E_4523	Aldehyde dehydrogenase family protein	4.3
T1E_4632	Amino acid ABC transporter permease protein	2.0
T1E_5295	AzlC-branched-chain amino acid transport protein AzlC	4.1
	Inorganic ion metabolism	
T1E_2123	Heme/hemin ABC transporter ATP-binding protein	2.0
T1E_2837	CBS domain protein	2.0
	Efflux pumps	
T1E_0242	TtgB-multidrug/solvent RND transporter TtgB	2.0
T1E_3785	Efflux transporter membrane fusion protein putative	2.8
	TtgG	4.8
	TtgH	4.7
	TtgI	4.5
	Energy production and carbohydrate metabolism	
T1E_1072	Aldehyde dehydrogenase family protein	3.1
T1E_1986	Gap-1-glyceraldehyde 3-phosphate dehydrogenase	2.9
T1E_1987	Edd-6-phosphogluconate dehydratase	4.9

T1E_1988	Glk-glucokinase	2.7
T1E_2001	2-keto-3-deoxy-6-phosphogluconate aldolase	2.3
T1E_2648	AceE-pyruvate dehydrogenase E1 component	2.6
T1E_2654	Major facilitator family transporter	3.7
T1E_2815	Sugar transferase putative	3.4
T1E_3265	FruK-1-phosphofructokinase	2.1
	Metal transport systems	
T1E_1068	Outer membrane ferric siderophore receptor putative	2.1
T1E_2193	ModR-molybdate transport regulator	2.0
T1E_2509	Outer membrane ferric siderophore receptor	2.1
T1E_5142	Outer membrane ferric siderophore receptor	2.2
T1E_5753	CopB-copper resistance protein B	2.0
	Oxygen stress	
T1E_0654	DnaK-dnaK protein	3.8
T1E_5239	AhpC-alkyl hydroperoxide reductase C subunit	2.0
	Regulatory proteins	
T1E_3670	DNA-binding response regulator	3.5
T1E_3973	Transcriptional regulator AraC family	3.3
T1E_3976	Transcriptional regulator LysR family	3.6
T1E_3993	PhoR-sensory box histidine kinase PhoR	2.4
T1E_5206	Transcriptional regulator LacI family	2.2
	TtgV	2.9
	Hypothetical proteins	
T1E_0256	Hypothetical protein	5.2
T1E_0617	Conserved hypothetical protein	2.6
T1E_3786	Conserved hypothetical protein	2.4

T1E_5141	Conserved hypothetical protein	3.2
T1E_5350	Conserved hypothetical protein	2.0

Table 5. *Pseudomonas putida* T1E-18 genes induced by the presence of 300 µM indole

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_0025	MvaB-hydroxymethylglutaryl-CoA lyase	2.0
T1E_2070	ABC transporter permease protein	2.3
T1E_3322	Ivd-isovaleryl-CoA dehydrogenase	3.6
T1E_3323	3-methylcrotonyl-CoA carboxylase beta subunit putative	3.0
T1E_3324	Enoyl-CoA hydratase putative	3.3
T1E_3325	Acetyl-CoA carboxylase biotin carboxylase putative	3.0
	DNA metabolism	
T1E_2166	DNA-binding protein Roi-related protein	2.2
	Efflux pumps	
	TtgG	3.3
	TtgI	3,1
	TtgH	2.9
T1E_5522	TtgE	2.6
	Energy production and conversion	
T1E_1050	Oxidoreductase putative	2.0
T1E_1262	Aminotransferase class III	2.1
T1E_1993	Sugar ABC transporter permease protein	2.0
T1E_3767	Beta-alanine-pyruvate transaminase	2.6
T1E_3768	MmsA-1-methylmalonate-semialdehyde dehydrogenase	2.0
T1E_5096	Major facilitator family transporter	2.3
T1E_5100	BkdA2-2-oxoisovalerate dehydrogenase beta subunit	2.0

T1E_5101	BkdB-2-oxoisovalerate dehydrogenase lipoamide acyltransferase	2.1
	Metal transport systems	
T1E_1542	Ferric siderophore ABC transporter periplasmic siderophore	2.1
	Regulatory proteins	
T1E_0145	Transcriptional regulator GntR family	2.0
T1E_1104	Transcriptional regulator LysR family	2.3
T1E_1849	Transcriptional regulator GntR family	2.3
T1E_5098	Transcriptional regulator BkdR family	2.0
	TtgV	3.1
	Lipid metabolism	
T1E_1788	Acyl-CoA dehydrogenase putative	2.3
	Hypothetical proteins	
T1E_1852	Conserved hypothetical protein	2.6

Suppl. Text

Response of *P. putida* T1E and T1E-18 to ampicillin

We explored the sets of genes upregulated and downregulated by 300 µg/ml ampicillin using gene expression microarrays (Suppl. Tables 2, 3 and 4). We found that among induced genes there were a number of transcriptional regulators belonging to the RpiR (T1E_2269), AmpR (T1E_0774), AsnC (T1E_2170), and LysR (T1E_0578) families; these are probably responsible for the changes in a limited set of approximately 56 genes that were induced >2-fold. We found that among the genes, with higher induction levels, there were three oxidoreductases (T1E_0851, T1E_5238 and T1E_0376), and T1E_2591, an iron-sulfur cluster-binding protein. Also in agreement with Fe-starvation is the induction of three membrane ferric siderophore receptors (T1E_2504, and T1E_3391). As expected from cell division arrest, the expression of one of the penicillin-binding proteins (T1E_3742) was also enhanced. In addition, there were a number of induced genes that encode proteins involved in cell-wall biosynthesis, lipoproteins and outer membrane turnover (i.e. T1E_0917, T1E_1888, T1E_1598 and T1E_3913) consistent with a potential role of ampicillin in inhibition of cell wall related processes. Gene expression changes in these cell envelope systems have been observed following oxidative damage with paraquat in *E. coli* (Pomposiello *et al.*, 2001). No genes related to oxidative stress were induced, although to overcame potential DNA damages. Regarding the central metabolism in response to ampicillin the cells activated a number of genes related to amino acid metabolism (glycine and tryptophane). Twenty genes, including some from the flagellar system and dipeptidases, were repressed more than 2-fold in response to ampicillin (Suppl. Table 2).

Since DOT-T1E-18 (Δ*ttgABC*) showed a marked sensitivity to ampicillin, we tested the effect of a sublethal concentration of this β-lactam (100 μg/ml for 3 h) on the global transcriptional pattern of DOT-T1E18 (Suppl. Table 3) (note that we could not use the same ampicillin concentration as in the parental strain because cells were not viable). We observed that in the TtgABC deficient DOT-T1E18 strain, a number of genes that were induced in the parental strain in response to ampicillin, were also induced in the mutant; including regulatory proteins (AmpR, T1E_0774 and the response regulator T1E_3731) and lipoproteins (T1E_0917, T1E_2185, T1E_2827). In addition a number of other genes were induced with ampicillin, including genes for alternative respiratory chains such as operons T1E_0717 through to T1E_0720 involved in the synthesis of alternative terminal oxidases of the respiratory chain, genes for arginine metabolism (T1E_1978, T1E_1979, T1E_2178 and T1E_2234). As observed in the wild-type iron-sulfur cluster proteins and Fe homeostasis transporters were also induced. (Suppl. Tables 3 and 4).

Suppl. Figure 1. HPF quenching in *P. putida* DOT-T1E and T1E-18 by indole and antibiotics.

(A, and B) Log change in colony-forming units per milliliter (cfu/ml). No-drug control is represented by grey triangles.

(C, D, E, F, G and H) HPF quenching. Representative measurements are shown and were taken 3 h following addition of drug.

(A, C, E and G) Survival (A) and HPF quenching in presence and absence of 300 µM indole and following exposure to bacteriostatic antibiotic (C) (4 µg/ml tetracycline [Tc], (E) (200 µg/ml erythromycin [Ery]) and bactericidal antibiotic (G) (1 µg/ml norfloxacin [Nor]) in *P. putida* DOT-T1E respectively.

(B, D, F and H) Survival (B) and HPF quenching in presence and absence of 300 µM indole and following exposure to bacteriostatic antibiotic (D) (1 µg/ml tetracycline [Tc], (F) (100 µg/ml erythromycin [Ery]) and bactericidal antibiotic (H) (0.5 µg/ml norfloxacin [Nor]) in *P. putida* DOT-T1E respectively.

Supp. Table 1. *Pseudomonas putida* DOT-T1E RND efflux pumps identified

Number	Annotation	RND efflux pump	Locus name
1	Acriflavine resistance protein	AcrB1	T1E_0107
	Efflux transporter, RND family, MFP subunit	AcrE1	T1E_0108
2	Toluene efflux pump outer membrane protein	TtgC	T1E_0241
	Toluene efflux pump membrane transporter	TtgB	T1E_0242
	Toluene efflux pump periplasmic linker protein	TtgA	T1E_0243
3	Hypothetical protein	ZncC	T1E_0620
	Periplasmic solute binding protein – zinc transport system	ZncA	T1E_0621
	ABC transporter related protein – zinc transport system	ZncB	T1E_0622
4	RND efflux system outer membrane lipoprotein	MacC	T1E_1280
	Macrolide export ATP-binding/permease protein	MacA	T1E_1281
	RND family efflux transporter MFP subunit	MacB	T1E_1282
5	Major facilitator transporter	TrpA	T1E_1585
	Secretion protein HlyD family protein	TrpB	T1E_1586
	RND efflux system outer membrane lipoprotein	TrpC	T1E_1587
6	Uncharacterized transporter HI0895 - Multidrug efflux RND transporter	UepB1	T1E_1916
	RND family efflux transporter MFP subunit	UepA1	T1E_1917
	Hypothetical protein	UepC1	T1E_1918
7	RND familyefflux transporter MFP subunit	MdtA	T1E_2276

	Multidrug resistance protein	MdtB	T1E_2277
	Multidrug resistance protein	MdtC	T1E_2278
	RND efflux system outer membrane lipoprotein	MdtD	T1E_2279
8	Arsenical pump membrane protein	ArsB	T1E_2721
	Regulatory protein	ArsR	T1E_2722
9	RND family efflux transporter MFP subunit		T1E_3612
	Acriflavin resistance protein	AcrB2	T1E_3613
10	RND efflux transporter	UepA2	T1E_3784
	Secretion protein HlyD family protein	UepB2	T1E_3785
11	Probable efflux pump outer membrane protein	SepC	T1E_4279
	Probable efflux pump membrane transporter	SepB	T1E_4280
	Probable efflux pump periplasmic linker protein	SepA	T1E_4281
12	RND family efflux transporter MFP subunit	AcrA	T1E_4452
	RND family efflux transporter MFP subunit	AcrE	T1E_4453
	Acriflavin resistance protein	AcrD	T1E_4454
13	Cation efflux system protein	CzcA1	T1E_4694
	Family cobalt/zinc/cadmium efflux transporter membrane fusion protein	CzcB1	T1E_4695
	Family cobalt/zinc/cadmium efflux outer membrane protein	CzcC1	T1E_4696
	Family heavy metal RND efflux protein – DNA binding heavy metal response regulator	CzcR1	T1E_4698
14	Acriflavin resistance protein	AcrB3	T1E_5088

	RND family efflux transporter MFP subunit		T1E_5089
15	Efflux transporter, RND family, MFP subunit	MexE	T1E_5217
	Probable aminoglycoside efflux pump	MexF	T1E_5218
	RND efflux system outer membrane lipoprotein	MexD	T1E_5219
16	Major facilitator transporter putative	CzcC2	T1E_5269
	Cobalt/zinc/cadmium resistance protein	CzcA2	T1E_5270
	RND family efflux transporter MFP subunit	CzcB2	T1E_5271
17	RND efflux system, outer membrane lipoprotein, NodT family	UepA3	T1E_5467
	Probable efflux pump membrane transporter	UepB3	T1E_5468
	Multidrug efflux pump membrane fusion protein	UepC3	T1E_5469
18	Toluene efflux pump outer membrane protein	TtgF	T1E_5521
	Toluene efflux pump membrane transporter	TtgE	T1E_5522
	Toluene efflux pump periplasmic linker protein	TtgD	T1E_5523
19	Toluene efflux pump outer membrane protein	TtgI	
	Toluene efflux pump membrane transporter	TtgH	
	Toluene efflux pump periplasmic linker protein	TtgG	

Suppl. Table 2. *P. putida* DOT-T1E genes repressed in presence of 300 µg/ml ampicillin

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_2744	Ribose-phosphate pyrophosphokinase family protein	-2.2
T1E_3356	4-hydroxyphenylpyruvate dioxygenase putative	-2.2
T1E_4648	Tabtoxinine-beta-lactam limiting dipeptidase putative	-2.0
	Energy production and conversion	
T1E_2172	Carbon-nitrogen hydrolase family protein	-2.6
T1E_2558	Oxidoreductase FMN-binding protein	-2.1
	Motility and secretion	
T1E_2334	Flagellar motor switch protein FliG	-2.8
T1E_3873	ClpP protease putative	-2.6
	DNA metabolism	
T1E_0528	ATPase AAA family	-2.2
T1E_4309	Urease accessory protein UreF	-2.0
	Regulatory proteins	
T1E_0510	Sensory box protein	-2.6
T1E_3238	Two-component sensor protein	-2.0
T1E_3447	Transcriptional regulator LysR family	-2.0
	Protein synthesis	
T1E_3848	Acetyltransferase GNAT family	-2.6
	Hypothetical proteins and unknown function proteins	

	Hypothetical protein-pWW0 17264-17719	-2.0
	xylR-pWW0	-3.8
T1E_0195	Conserved hypothetical protein	-3.2
T1E_0913	Hypothetical protein	-2.9
T1E_1729	Hypothetical protein	-2.1
T1E_2380	Hypothetical protein	-3.3
T1E_3087	Phage FluMu protein gp38	-2.2
T1E_4078	Conserved hypothetical protein	-2.0
T1E_5002	Conserved hypothetical protein	-2.2

Suppl. Table 3. *Pseudomonas putida* T1E-18 genes induced in presence of 100 µg/ml ampicillin

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_1978	ArcA-arginine deiminase	3.9
T1E_1979	ArcD-arginine/ornithine antiporter	5.2
T1E_2178	Prolyl oligopeptidase family protein	2.1
T1E_2234	Hydrolase haloacid dehalogenase-like family	2.1
	DNA metabolism	
T1E_0743	XdhA-xanthine dehydrogenase XdhA subunit	3.2
	Inorganic ion metabolism	
T1E_1982	HemO-heme oxygenase	2.6
	Energy production and conversion	
T1E_0427	Oxidoreductase putative	2,1
T1E_0717	CcoN-1-cytochrome c oxidase cbb3-type subunit I	3.6
T1E_0718	CcoO-1-cytochrome c oxidase cbb3-type subunit II	2.9
T1E_0719	CcoQ-1-cytochrome c oxidase cbb3-type CcoQ subunit	3.7
T1E_0720	CcoP-1-cytochrome c oxidase cbb3-type subunit III	3.9
T1E_0730	HemN-oxygen-independent coproporphyrinogen III oxidase	2.7
T1E_0952	HprA-glycerate dehydrogenase	2.0
T1E_2621	Azurin	2.6
T1E_4339	PetC-ubiquinol-cytochrome c reductase cytochrome c1	2.3
	Metal transport systems	

T1E_2559	Iron-sulfur cluster-binding protein	2.5
T1E_3391	Outer membrane ferric siderophore receptor	6.9
	Regulatory proteins	
T1E_0387	Universal stress protein family	2.4
T1E_0774	Transcriptional regulator AmpR putative	4.7
T1E_0804	Transcriptional regulator GntR family	2.0
T1E_3731	Response regulator	2.1
T1E_4922	Transcriptional regulator AsnC family	3.2
	Lipid and membrane proteins	
T1E_0917	Lipoprotein putative	2.0
T1E_2185	Acyl-CoA dehydrogenase putative	7.0
T1E_2827	Surface colonization protein putative	3.9
T1E_3506	OprG-outer membrane protein OprG	3.5
T1E_3957	Long-chain acyl-CoA thioester hydrolase family protein	2.0
T1E_3965	AccC-2-acetyl-CoA carboxylase biotin carboxylase	2.1
	Hypothetical proteins	
T1E_0217	Conserved hypothetical protein	2.0
T1E_0716	Conserved hypothetical protein	2.0
T1E_1884	Hypothetical protein	3.3
T1E_1975	Conserved hypothetical protein	2.8
T1E_2534	Conserved hypothetical protein	2.0
T1E_3782	Conserved hypothetical protein	2.0

Suppl. Table 4. *P. putida* T1E-18 repressed genes in presence of 100 µg/ml ampicillin

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_2860	Polyamine ABC transporter permease protein	-2,3
	Secretion	
T1E_1854	SEC-C domain protein	-2,1
	Inorganic ion metabolism	
T1E_0566	CysNC-sulfate adenylyltransferase subunit 1/adenylylsulfate	-2,0
T1E_4626	TauB-taurine ABC transporter ATP-binding protein	-2,0
	Energy production and conversión	
T1E_0642	TpiA-triosephosphate isomerase	-2,0
T1E_4171	Ferredoxin 2Fe-2S	-2,0
	Regulatory proteins	
T1E_1941	NusG-transcription antitermination protein NusG	-2,0
T1E_4800	Transcriptional regulator AraC family	-2,2
T1E_5327	Sensory box histidine kinase/response regulator	-2,0
	Protein synthesis	
T1E_0711	PPsD-non-ribosomal siderophore peptide synthetase	-2,1
	Translation and ribosomal structure proteins	
T1E_0159	RpmE-ribosomal protein L31	-2,0
T1E_0533	InfA-translation initiation factor IF-1	-2,3
	Lipid and membrane proteins	

T1E_1294	AcpP-acyl carrier protein	-2,0
T1E_1837	Membrane protein putative	-2,1
T1E_2003	Peptidase M23/M37 family	-2,1
T1E_4277	N-acetylmuramoyl-L-alanine amidase putative	-2,0
	Hypothetical and unknown function proteins	
T1E_0408	Conserved hypothetical protein	-2,1
T1E_0640	Conserved hypothetical protein	-2,2
T1E_1518	Conserved hypothetical protein	-2,6
T1E_1803	Conserved hypothetical protein	-2,3
T1E_1860	Conserved hypothetical protein	-2,0
T1E_3240	Conserved hypothetical protein	-2,0
T1E_3330	Hypothetical protein	-2,4
T1E_3795	BNR domain protein	-2,0
T1E_4172	Conserved hypothetical protein	-2,0
T1E_5189	Hypothetical protein	-2,1
T1E_5330	Conserved hypothetical protein	-2,8

Suppl. Table 5. *P. putida* DOT-T1E repressed genes in the presence of 300 μM indole

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_1260	Conserved hypothetical protein	-2.7
T1E_2403	AstB-succinylarginine dihydrolase	-4.0
T1E_2407	ArgD-acetylornithine aminotransferase	-2.4
T1E_3495	AnsB-L-asparaginase type I	-3.1
T1E_4051	Basic amino acid ABC periplasmic transporter	-2.0
	Motility and secretion	
T1E_2341	FliS-flagellar biosynthetis protein FliS	-2.2
	DNA metabolism	
T1E_5744	XseB-exodeoxyribonuclease VII small subunit	-2.3
	Inorganic ion metabolism	
T1E_4720	Bfr-bacterioferritin	-2.2
	Energy production and conversion	
T1E_1262	Aminotransferase class III	-2.4
T1E_1557	HmgA-homogentisate 1 2-dioxygenase	-14.1
T1E_1558	Fumarylacetoacetase	-3.5
T1E_2404	AruD-succinylglutamic semialdehyde dehydrogenase	-3.1
T1E_2485	HutI-imidazolonepropionase	-2.9
T1E_2707	Citrate MFS transporter putative	-3.5
	Regulatory proteins	

T1E_1259	Transcriptional regulator putative	-6.7
T1E_4055	PhhR-sigma-54 dependent transcriptional regulator	-3.7
	Lipid and membrane proteins	
T1E_3598	3-hydroxyacyl-CoA dehydrogenase family protein	-2.2
T1E_3965	AccC-2-acetyl-CoA carboxylase biotin carboxylase	-3.4
	Hypothetical proteins	
T1E_0070	Conserved hypothetical protein	-2.7
T1E_0716	Conserved hypothetical protein	-2.0
T1E_2402	Conserved hypothetical protein	-2.6
T1E_2456	Conserved hypothetical protein	-2.3
T1E_3807	Conserved hypothetical protein	-2.1

Suppl. Table 6. *P. putida* T1E-18 repressed genes in presence of 300 μM indole

Identifier	Description	Fold Change
	Amino acid metabolism	
T1E_2524	Amino acid ABC transporter periplasmic amino acid-binding protein	-5.5
	DNA and RNA metabolism	
T1E_0182	RNA methyltransferase TrmH family group 2	-2.0
T1E_3033	ISPpu13 transposase Orf2	-2.3
T1E_3978	PurE-phosphoribosylaminoimidazole carboxylase	-2.4
	Inorganic ion metabolism	
T1E_0197	Na+/H+ antiporter putative	-2.8
	Regulatory proteins	
T1E_2026	Transcriptional regulator LysR family	-2.0
T1E_2398	CsrA-carbon storage regulator CsrA	-2.8
T1E_3976	Transcriptional regulator LysR family	-2.2
	Lipid and membrane proteins	
T1E_2815	Sugar transferase putative	-2.0
T1E_5109	Membrane protein putative	-2.6
T1E_5220	Lipase GDSL family	-2.1
T1E_5687	Membrane protein MviN family	-2.5
	Hypothetical proteins	
T1E_2619	Conserved hypothetical protein	-3.5
T1E_5665	Conserved hypothetical protein	-2.2

Suppl. Table 7. Oligo sequences used in qRT-PCR assays in *P. putida* DOT-T1E

Ampicillin assays		Indole assays	
Name	**Sequence 5'-3'**	**Name**	**Sequence 5'-3'**
T1E_1753F	CGAATCGAAATGCCCGTTCC	**T1E_4523F**	CGAAGGCGCGAAGGTTTCCTT
T1E_1753R	TCATCAGGGCGGTCAGGTCTTT	**T1E_4523R**	CATCCAGGGCCTTCTCGATGTC
T1E_3479F	GAGCAAGATTCTCACCACCG	**T1E_5295F**	CCGCCAAGCCTTTCTTCACG
T1E_3479R	CCCGAGCCTTTGGCATGGA	**T1E_5295R**	AGCATGCCAATGGCCACCAG
T1E_4765F	TGACAGGGCCAACACCAATGC	**T1E_1987F**	CATCCGCGCATCCTTGAGGT
T1E_4765R	GGGTGATTTTCTCGCGCATGA	**T1E_1987R**	AGAGTCTGCTTGTCTTCGCTGCC
T1E_5238F	TTGGACGCCACGCTTAAATCG	**T1E_5142F**	TGTGAGTTCGCCACGCCTGAT
T1E_5238R	ACCGTCCGCGCTGAAGGTAA	**T1E_5142R**	GCGGCCTTGTCCACCTTGTAAT
T1E_4980F	CGACAACAAGAAGCGCGCCT	**T1E_0654F**	CCACCAACTCGTGCGTCTCCA
T1E_4980R	CGACGATACGGCCTTTTGGC	**T1E_0654R**	GTGTTGTGCGGGTTGGTGACC
T1E_1218F	TTGAAACTGACGCCACGCCA	**T1E_0256F**	CGAGCTCAGGCTTGGCACACTA
T1E_1218R	GTCATTTCGATCGCGCCCTT	**T1E_0256R**	ACCGACGATGGCATTGAACGG
		TTGGF	CGTTGGCGGTGCTGCTTGT
		TTGGR	CTGCGCCTGCACGGTGTAGA

qRT-PCR DOT-T1E Ampicillin 300 µg/ml

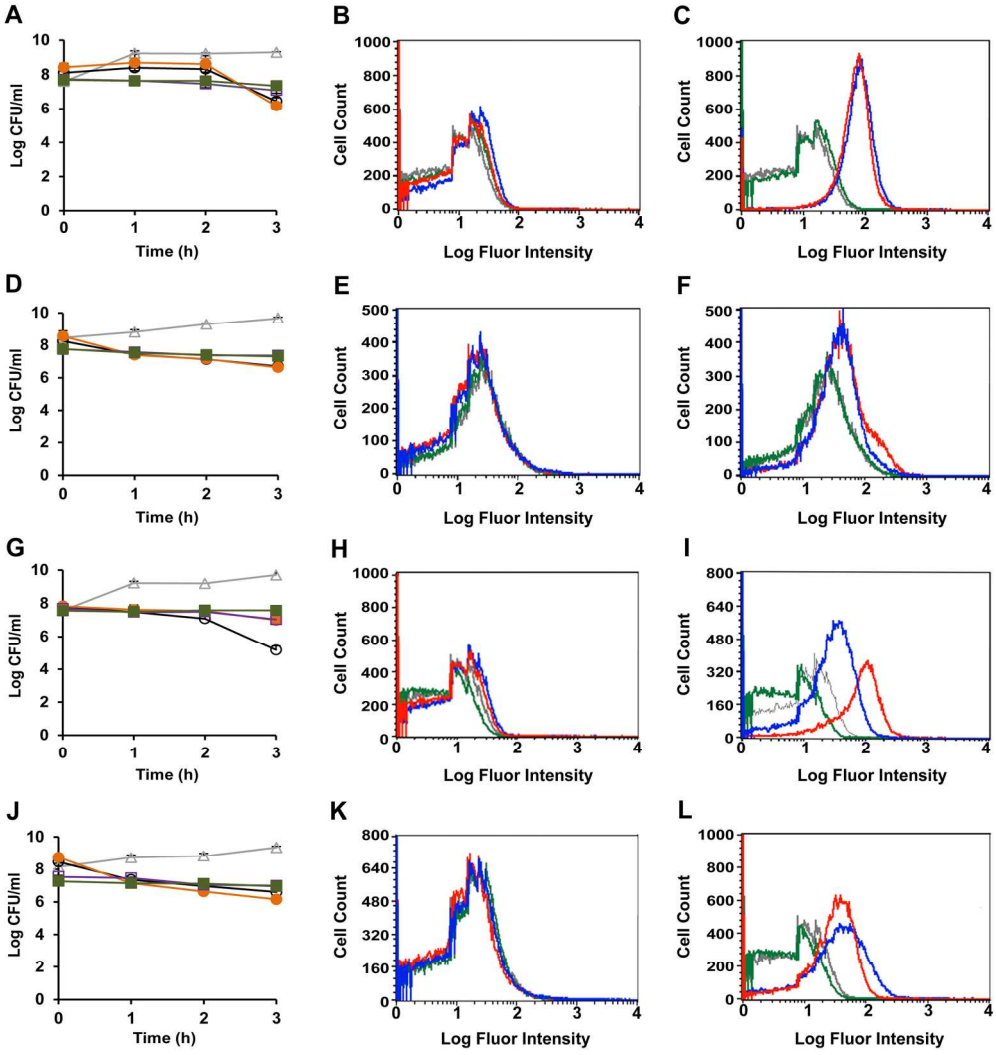

Figure 1
180x192mm (300 x 300 DPI)

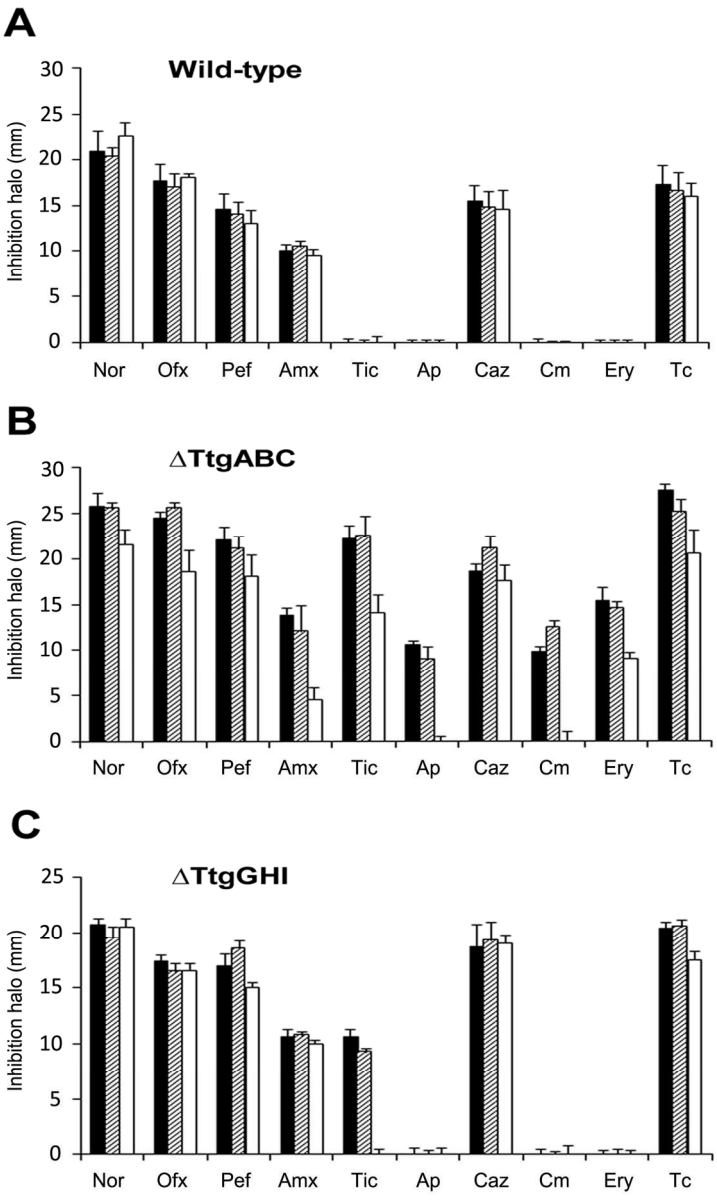

Figure 2
128x204mm (300 x 300 DPI)

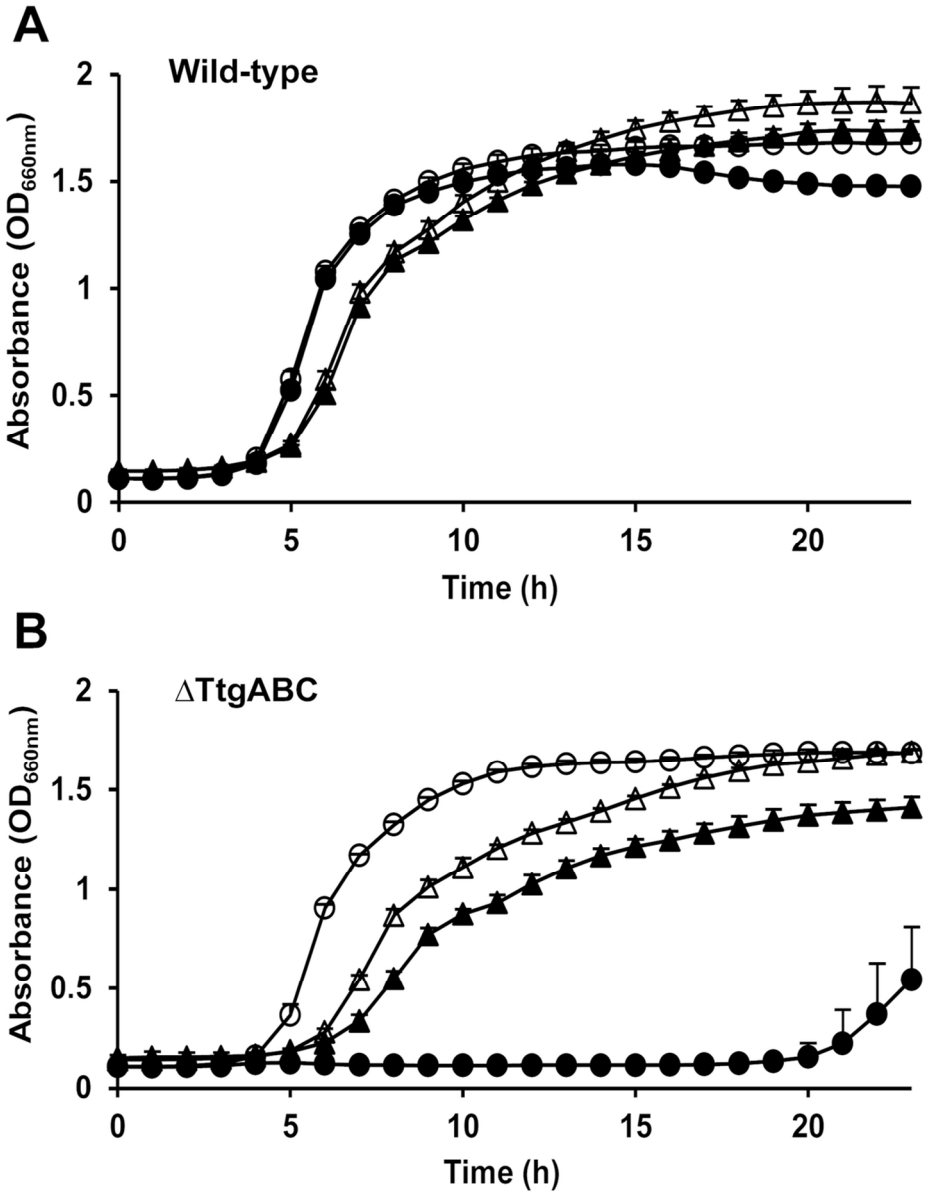

Figure 3
103x133mm (300 x 300 DPI)

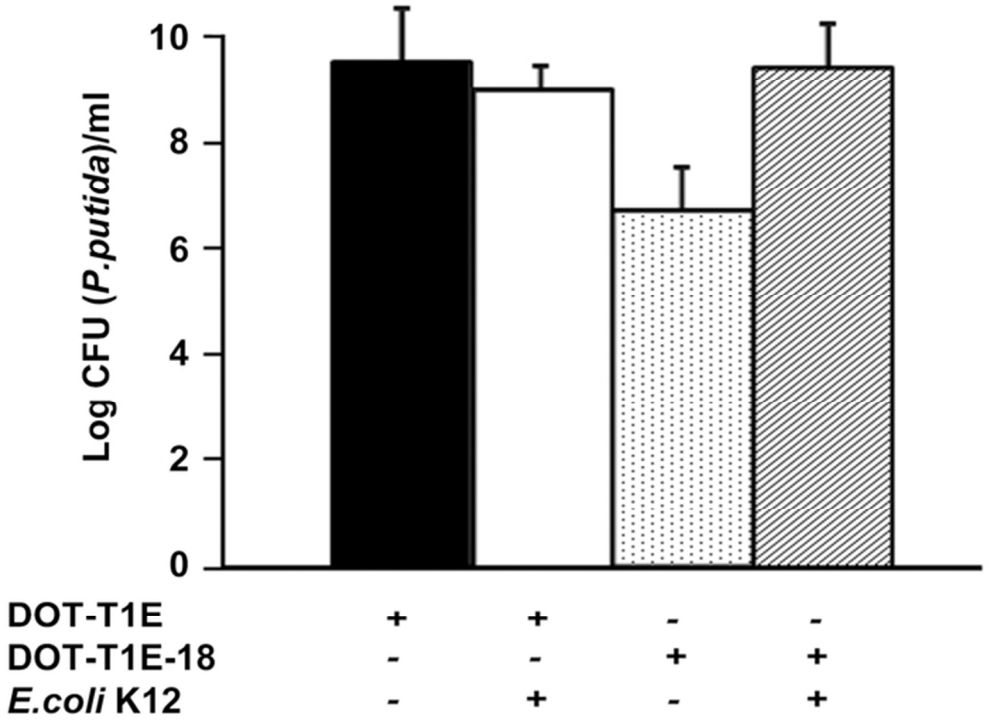

Figure 4
59x44mm (300 x 300 DPI)

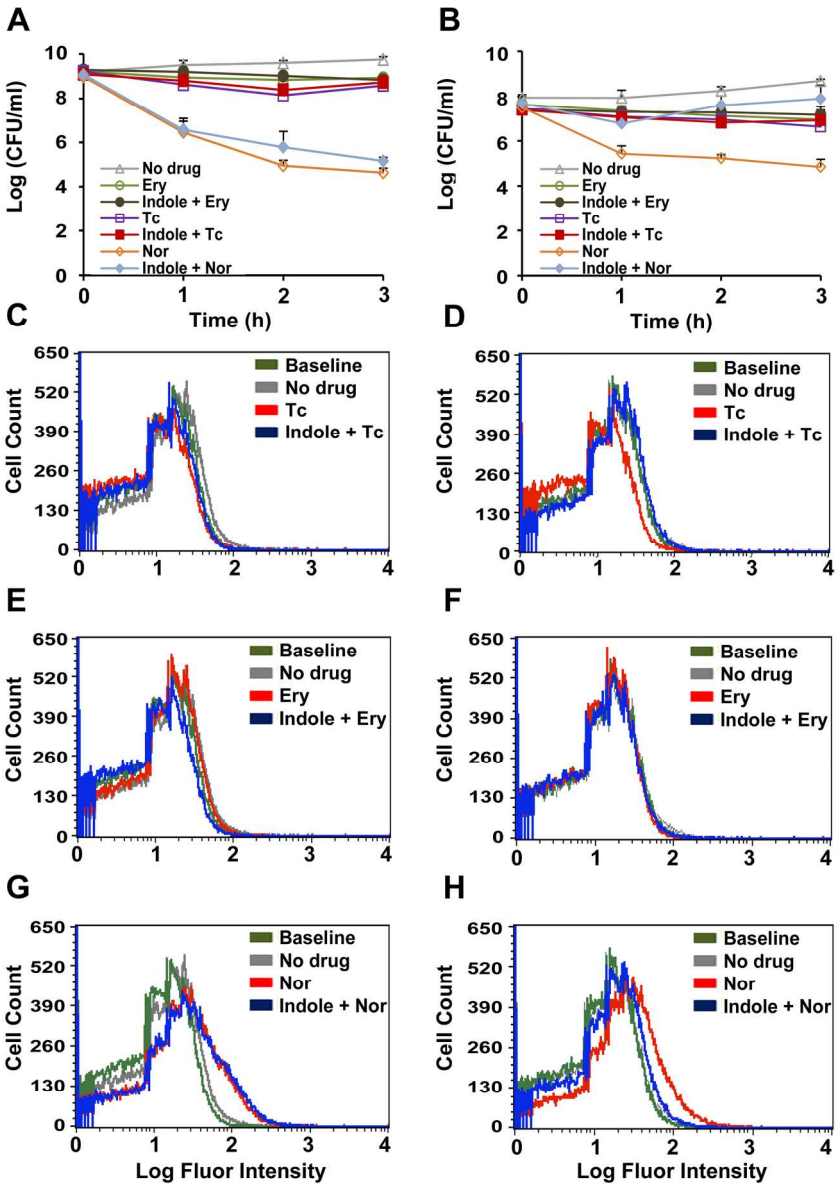

Figure S1
160x213mm (300 x 300 DPI)