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Desarrollo de procesos biotecnológicos basados en cepas de *Pseudomonas putida*. Mecanismos de señalización y biosíntesis de antibióticos

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Granada, 24 de septiembre de 2014

Director de la Tesis	Doctorando
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“Un experto es aquel que sabe cada vez más sobre menos cosas
hasta que sabe absolutamente todo acerca de nada…”
Weber

“Cuando descubres algo tú mismo,
incluso si eres la última persona en La Tierra en ver la luz,
nunca lo olvidarás.”
Carl Sagan

A mis padres,
A mi hermano,
A Carla

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List of abbreviations

ΔG	Gibbs free energy
ΔH	Change in enthalpy
ΔS	Change in entropy
ABC	ATP-binding cassette
Amx	Amoxicillin
Ap	Ampicillin
bp	Base pair
Caz	Ceftazidime
CD	Circular dichroism
CDS	Coding DNA sequence
CFA	Cyclopropane fatty acid
CFU	Colony forming units
Cip	Ciprofloxacin
Cm	Chloramphenicol
CTAB	Cetyltrimethylammonium bromide
Da	Dalton
DSC	Differential scanning calorimetry
EDTA	Ethylene diamine tetraacetic acid
Ery	Erythromycin
Gm	Gentamicin
HPF	3-p-(hydroxyphenyl) fluorescein
HPLC	High performance liquid chromatography
HTH	Helix-turn-helix
ITC	Isotermal titration calorimetry
K_A	Association constant
kb	Kilobase
K_D	Dissociation constant
Km	Kanamycin
LB	Luria-Bertani media
LC	Liquid cromatography
Mar	Multiple antibiotic resistance
MATE	Multidrug and toxic-compound extrusion
MDR	Multidrug resistance

MFS	Major facilitator superfamily
MRP	Multidrug resistance protein
MS	Mass spectrometry
Nal	Nalidixic acid
Nor	Norfloxacin
N-terminal	Amino terminal
O.D.	Optical density
ORF	Open reading frame
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEF	Pefloxacin
PIPES	1,4-Piperazinediethanesulfonic acid
Qac	Quaternary ammonium compounds
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rif	Rifampicin
RND	Resistance nodulation cell-division
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
SMR	Small multidrug resistance
Sp	Spectinomycin
sRNA	Small ribonucleic acid
Tc	Tetracycline
TCA	Tricarboxylic acid
Tic	Ticarcillin
Ttg	Toluene tolerance gene
WT	Wild-type

RESUMEN

El incremento de la resistencia a antibióticos por parte de las bacterias y el desarrollo de bacterias multi-resistentes a distintos biocidas están dando lugar a infecciones crónicas que, en pacientes inmunodeprimidos y personas de avanzada edad, pueden representar serios riesgos para su salud. Esto ha despertado un creciente interés en el mundo científico por el estudio de los mecanismos de resistencia a antibióticos, así como la búsqueda de nuevas moléculas bioactivas en la lucha frente a las infecciones microbianas. Las bacterias incrementan su resistencia a compuestos antimicrobianos debido a varios mecanismos como son la resistencia adquirida mediante mutaciones y la transferencia horizontal de genes, la resistencia adaptativa debido a una mayor expresión génica en respuesta a cambios ambientales, y la resistencia intrínseca motivada por bombas de extrusión y elementos de la membrana lipídica. Estos mecanismos, de manera individual o actuando simultáneamente, dan lugar a que las bacterias patógenas cada vez sean más difíciles de controlar y erradicar, lo que finalmente podría derivar en un problema sin precedentes para la humanidad. La búsqueda de nuevos compuestos antimicrobianos, los cuales apenas se han obtenido desde el la edad de oro de los antibióticos entre 1940 y 1960, es de vital importancia para poder luchar frente a estas bacterias multi-resistentes.

Pseudomonas putida DOT-T1E es una bacteria que se aisló de una planta de tratamiento de aguas residuales en Granada, caracterizada por ser capaz de crecer en presencia de altas concentraciones de disolventes tóxicos y antibióticos. Esto la convierte en una bacteria clave para el estudio de los mecanismos de resistencia a este tipo de compuestos, a pesar de no ser una bacteria patógena para humanos. Entre sus principales mecanismos de resistencia a este tipo de compuestos destacan las bombas de tipo RND. Estas bombas están formadas por tres proteínas que crean un canal desde el interior celular hasta el exterior, lo que permite a la bacteria la extrusión al medio externo de los compuestos nocivos. De las 21 bombas de tipo RND que presenta la cepa DOT-T1E, las principales bombas de extrusión son las llamadas TtgABC, TtgDEF y TtgGHI. TtgABC es la bomba fundamental en la extrusión de antibióticos, mientras que TtgDEF y TtgGHI son las encargadas de la expulsión de disolventes desde el interior de la célula hacia el medio. TtgABC está regulada por TtgR, mientras que TtgDEF y TtgGHI están reguladas por TtgV. Ambos reguladores se sintetizan constitutivamente reprimiendo la expresión de los genes de las bombas. En presencia de efectores, el represor sufre un cambio conformacional y de esta

forma se libera del promotor de las bombas, lo que permite incrementos en los niveles de expresión de los genes y un aumento concomitante de la resistencia de la bacteria al efector o agente inductor.

El trabajo experimental del primer y segundo capítulo de esta Tesis Doctoral persigue identificar residuos clave en la transmisión de la señal intramolecular en el regulador transcripcional TtgV, y estudiar el papel del indol en la resistencia a antibióticos de *P. putida* DOT-T1E, así como su efecto como molécula de señalización inter-especie. TtgV es un regulador que se había cristalizado y caracterizado previamente en nuestro grupo, si bien el papel de algunos aminoácidos claves en la estructura tridimensional no se había analizado en detalle. Mediante técnicas de mutagénesis se han generado mutantes y mediante ensayos de calorimetría de titulación isotérmica (ITC) se ha analizado el papel de esos residuos en la función del represor. En este trabajo hemos estudiado el papel clave del residuo Q86 viéndose que este residuo es de vital importancia para la transmisión de la señal entre el dominio de reconocimiento de ligando y el dominio de unión a ADN. También hemos estudiado otros aminoácidos claves relacionados con la unión a ADN, como son los residuos en las posiciones 19, 35, 44 y 46. Mutaciones en los aminoácidos de las posiciones 19 y 35, fuera del dominio HTH de unión a ADN, han demostrado que estos residuos fuera del HTH son relevantes para el reconocimiento del ADN por parte de TtgV, un hecho inusual en reguladores transcripcionales que establecen contactos y reconocen sus operadores fundamentales a través del dominio HTH.

El segundo capítulo se ha centrado en el estudio de la resistencia a antibióticos bactericidas y bacteriostáticos por *Pseudomonas putida* DOT-T1E y el papel en la misma por parte de TtgGHI, bomba de extrusión regulada por la acción de TtgV, un regulador transcripcional que reconoce indol como efector. Estudios de transcriptómica han puesto de manifiesto que el indol incrementa la expresión de TtgGHI lo que provoca un aumento de la resistencia a antibióticos de *P. putida* DOT-T1E cuando la principal bomba relacionada con la extrusión de antibióticos, TtgABC, está truncada. Este resultado demuestra un papel secundario de TtgGHI en la extrusión de compuestos antimicrobianos. Diversas enterobacterias son capaces de producir y excretar indol al medio. Co-cultivos de *E. coli* y variantes génicas de *P. putida,* así como estudios de transcriptómica, han permitido demostrar un papel de TtgV en la comunicación inter-bacteriana, debido a que el

indol producido por otras bacterias puede ser detectado por este represor y utilizado para responder a diversos estreses en *P. putida* DOT-T1E.

En el tercer capítulo se ha abordado el objetivo de revelar los mecanismos de resistencia a diversos antibióticos de *P. putida* DOT-T1E mediante ensayos de transcriptómica a nivel global utilizando técnicas de secuenciación masiva de ARN. Esta aproximación ha permitido obtener una gran cantidad de información acerca de cómo DOT-T1E responde a distintos antibióticos. Estos análisis han confirmado los mecanismos de acción descritos para diversos antibióticos mediante la identificación de un incremento en la expresión de genes relacionados con cada una de las funciones que inhiben, y además, han revelado nuevos genes que podrían estar actuando como respuesta frente a estos estreses. Análisis a nivel funcional han determinado que, si bien la mayor parte de antibióticos inducen la expresión de funciones como actividad catalítica, unión a ligandos y procesos metabólicos entre otros, los genes que se inducen en respuesta a cada antibiótico suelen ser distintos. Dentro del programa transcripcional en respuesta a antibióticos hemos detectado 138 nuevos ARN no codificantes, lo que aumenta los 16 previamente identificados hasta ahora, presentando perfiles de expresión muy similares a los descritos para los ARN mensajeros en presencia de antibióticos. Los resultados obtenidos muestran además la modulación de la expresión de una gran cantidad de genes con función desconocida hasta el momento, lo que pone de manifiesto la necesidad de profundizar en el estudio de los mecanismos de acción de los antibióticos con el objetivo de poder combatir de forma más eficiente las bacterias multi-resistentes.

En los capítulos cuatro y cinco se decidió tratar la aplicación biotecnológica de una cepa de *P. putida* DOT-T1E mutada en la principal bomba de extrusión de antibióticos como bacteria modelo en el descubrimiento de nuevos compuestos con actividad antimicrobiana. Igualmente, estos capítulos se centran en el aislamiento de una nueva cepa de *Pseudomonas* capaz de producir una familia de moléculas nuevas, así como el estudio del efecto de estos compuestos como antimicrobianos y antifúngicos.

La búsqueda de nuevos compuestos naturales bioactivos es una prioridad en la lucha frente a bacterias patógenas. El desarrollo de una nueva plataforma de búsqueda de bacterias productoras de compuestos antimicrobianos, usando como bacteria control una cepa de *P. putida* DOT-T1E más sensible a antibióticos, ha permitido aislar diversas bacterias productoras de compuestos conocidos y

desconocidos con actividad antimicrobiana. Se ha seleccionado una de ellas, *Pseudomonas* sp. 250J, que produce xantolisinas, un nuevo tipo de lipodepsipéptidos cíclicos con actividad antimicrobiana y antifúngica además de mostrar actividad sinérgica cuando se aplica junto con otros antibióticos. La secuenciación del genoma de 250J ha permitido identificar los genes de síntesis de estos compuestos así como otros genes relacionados con la síntesis de metabolitos secundarios, entre los cuáles se han encontrado genes relacionados con la producción de bacteriocinas, entre otros. Además, hemos podido identificar una serie de genes relacionados con la patogenicidad potencial de esta bacteria.

En definitiva, con esta Tesis Doctoral se han pretendido analizar y aclarar diversos mecanismos relacionados con la resistencia a antibióticos de la bacteria *Pseudomonas putida* DOT-T1E, así como desarrollar una nueva plataforma la cual permita una búsqueda eficiente de nuevos compuestos antimicrobianos que ayuden en la lucha frente a las, cada vez más frecuentes, bacterias multi-resistentes.

I. GENERAL INTRODUCTION

1. *Pseudomonas putida*

Bacteria of the genus *Pseudomonas* were first described at the end of the 19th century by Migula (1894) and are represented by a large number of species that belong to the γ-proteobacteria class. They are Gram-negative, rod-shaped, aerobic and motile due to the presence of one or several polar flagella (Palleroni, 1984) (Figure 1). Bacteria of this genus are widespread in the environment since they are present in a wide diversity of habitats. For instance, some strains of the *P. aeruginosa* species are frequent colonizers of the human lungs where they are able to form biofilms in cystic fibrosis patients; *P. syringae* strains are frequent plant pathogens. In contrast with these pathogenic species, most of *Pseudomonas* species usually are non-pathogenic bacteria, and play relevant roles in recycling nutrients in the environment, and a few of them have been described as able to promote plant growth. In this regard, some strains are able to proliferate on plant surfaces (*P. fluorescens*) or in soil located on the surface of plant roots and even on plant leaves (*P. putida*). These microbes can also act as biocontrol agents against plant pathogens (Costerton, 1999; Rainey, 1999; Ramos-González *et al.*, 2005; Setubal *et al.*, 2005).

Pseudomonas putida strains are able to colonize the root systems of a large number of plants, establishing and persisting in the rhizosphere at a relatively high cell density (Molina *et al.*, 1998; Ramos *et al.*, 2002; Duque *et al.*, 2013). Strains of this species are also able to attach to plant surfaces and to form biofilms on them, which facilitates colonization and confer resistance to biotic and abiotic stresses (Yousef-Coronado *et al.*, 2008).

Pseudomonas species are considered bacteria that are able to adapt and survive in different ecological niches. This capacity can be explained by their large genomes, which often exceed 6 Mb. The first sequencing project of *Pseudomonas* genome was completed in 2000 with *P. aeruginosa* PAO1 strain (Stover *et al.*, 2000); what revealed that its size was comparable to that of a simple eukaryote like *Saccharomyces cerevisiae*. The genome of the soil bacterium *P. putida* KT2440 was sequenced by a German-American consortium providing new information related with the ubiquitous location in the environment of this group of microbes (Nelson *et al.*, 2002). The genome structure allowed identifying gene islands, mobile elements, phages and transposons revealing the high capacity of *P. putida* strains to acquire new genetic information via horizontal gene transfer. The genome sequence of at least other 9 strains of *P. putida* has been deciphered and

this helped to define the pan-genome structure of the species. *Pseudomonas putida* strains present around 5500 genes of which the core genome has been estimated in 3500 genes (Zulema Udaondo and Juan L. Ramos, unpublished results). Almost half of the genes have unknown functions, while of those with an assigned function, a high proportion correspond to genes that encode for transporters and transcriptional regulators.

Genome sequencing is continuously increasing the knowledge of these strains and it can explain their ability to adapt and survive in different environments and to use biogenic and xenobiotic compounds (Timmis *et al*., 1988; Ramos *et al*., 1994; Matilla *et al*., 2011; Tao *et al*., 2011; Udaondo *et al*., 2013). Strains of *P. putida* are a paradigm of metabolically versatile microorganisms being able to use lineal and aromatic hydrocarbons as carbon and as energy sources and many inorganic and organic nitrogen sources (Udaondo *et al*., 2013). The information derived from genome analysis provides an interesting tool for microbiological and biotechnological research in this species, which together with the easy genetic manipulation of *P. putida*, make this species a model to study bioremediation, biocatalysis and phenomena related with root colonization (Udaondo *et al*., 2012; Segura and Ramos, 2013).

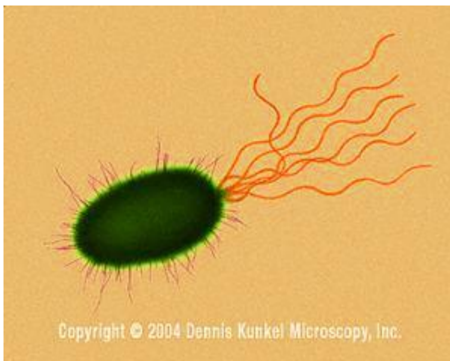

Figure 1. TEM image of *Pseudomonas* sp. Their dimensions range between 0.5 and 1.0 μm x 1.5 to 4 μm. *Photographer: Dennis Kunkel.*

A relevant feature is that some strains of the *P. putida* species are able to survive and adapt to hostile environments, such as soils polluted with high concentrations of organic solvents and toxic aromatic hydrocarbons. In fact, *P. putida* strains like DOT-T1E, S12, GM1, F1 or Idaho could be considered as extremophiles due to their solvent-tolerant characteristics, what allow them to thrive in the presence of high concentrations of toxic solvents such as *p*-xylene,

styrene, octanol, and toluene (Cruden *et al.*, 1992; Ramos *et al.*, 1995; Isken and de Bont, 1996; Huertas *et al.*, 1998; Segura *et al.*, 2012; Udaondo *et al.*, 2012).

1.1 *Pseudomonas putida* DOT-T1E

One of the *P. putida* solvent-tolerant strains with capacity to grow in the presence of toxic compounds is *P. putida* DOT-T1E. This strain was isolated in 1995 from a wastewater treatment plant in Granada (Ramos *et al.*, 1995) based on its capacity to thrive in the presence of high concentrations of toluene. Initial studies showed that when DOT-T1E was exposed to 1% (vol/vol) toluene – a concentration that kills all the cells of a culture of *E. coli* with 10^9 CFU/ml -, the number of viable cells decreased by 4 to 5 order of magnitude, but the set of survivors were able to grow colonizing the polluted niche (Huertas *et al.*, 1998). DOT-T1E is also able to use toluene as a carbon and energy source through its degradation by the TOD pathway (Gibson *et al.*, 1970; Mosqueda *et al.*, 1999). The genes of this pathway are organized in two adjacent transcriptional units, *todXFC1C2BADEGIH* and the genes encoding the corresponding two-component regulatory system, *todST*, which form an independent operon that is expressed constitutively (Busch *et al.,* 2007). The activation of this catabolic pathway is mediated by the TodS/TodT two-component system in response to toluene and other aromatic hydrocarbons, thus regulating the expression of the promoter termed P_{todX}, located upstream from the *todX* gene (Lau *et al.*, 1997; Lacal *et al.*, 2008; Busch *et al.*, 2010; Krell *et al.*, 2012).

Pseudomonas putida DOT-T1E genome was recently sequenced using the *454* technology and it has helped to identify potential solvent tolerance clusters responsible for the enhanced solvent-tolerance of this strain over other *P. putida* strains (Udaondo *et al.*, 2013). The 6.39 Mbp genome of *P. putida* strain DOT-T1E comprises two circular replicons: a single chromosome of 6,260,702 bp (G+C content of 63%) and a 133,451 bp self-transmissible plasmid named pGRT1 present in cells at a copy number of one plasmid per chromosome. The pGRT1 plasmid encodes one hundred and twenty six proteins, which represent less than 2% of the total number of encoded proteins, and has a slightly lower G+C content (58%) than the chromosome, but a similar coding density. The chromosome shows 5756 open reading frames, 58 tRNAs and 170 unique CDS that share no similarity with CDS present among the sequenced *Pseudomonas* genomes.

2. Mechanisms of solvent tolerance

Solvents are characterized by its capacity to cross rapidly the cellular envelope of Gram-negative bacteria and to accumulate in the cytoplasmic membrane (Ramos *et al.*, 2002). Gram-negative bacteria in general, and *P. putida* strains in particular, use several mechanisms to avoid the entry of solvents (Segura *et al.*, 2012), such as adaptive changes in membrane, chaperones to refold denatured proteins, degradation pathways and efflux pumps, last two focused in decreasing the load of solvents (Figure 2).

2.1 Membrane modifications

The first resistance line of *P. putida* strains to toxic compounds present in the environment takes place at the membrane that is composed of a phospholipid bilayer, which in the presence of aromatic compounds increases its fluidity. The contact solvent-membrane initiates two different responses. In the short term, an isomerization of *cis* unsaturated fatty acids to *trans* unsaturated fatty acids occur, a reaction mediated by the *cis-trans* isomerase (Heipieper *et al.*, 2003) that lead to higher density of membranes, thus reducing the entry of solvents what finally allow cells to adapt immediately to the new environmental conditions. The long-term response at the membrane level is produced with a change in the saturated to unsaturated fatty acid ratio (Pinkart and White, 1997), and in other cases the length of the acyl-chains and phospholipids head group modifications, providing a denser membrane packing and contributing to solvent tolerance (Segura *et al.*, 2012).

The cyclopropane fatty acids (CFAs) formation has also been proposed to occur in response to environmental stresses (Ramos *et al.*, 1997; Bernal *et al.*, 2007). By the action of the cyclopropane synthases, expressed in the late-exponential and early-stationary phases, *cis* unsaturated fatty acids are converted in CFA by the addition of a methylene group across the double bond of the unsaturated fatty acids (Grogan and Cronan, 1997; Muñoz-Rojas *et al.*, 2006) what counteracts the increased membrane fluidity caused by solvent alteration and increasing the membrane packaging density. As evidence of this process, a cyclopropane synthase mutant of *P. putida* DOT-T1E, *cfaB*, showed to be more sensitive to a toluene shock than the parental strains (Pini *et al.*, 2009).

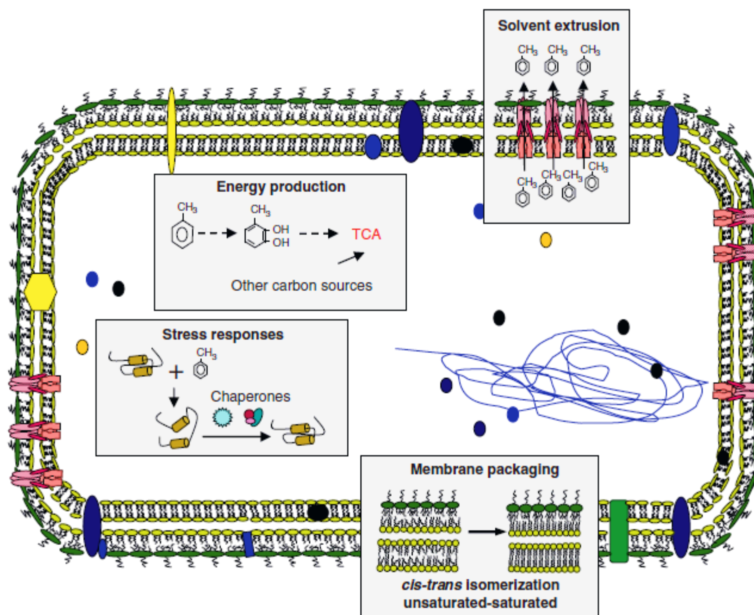

Figure 2. Schematic representation of the main mechanisms involved in the multifactoral solvent tolerance process in several microorganisms (Segura *et al.*, 2012).

2.2 Chaperones

In spite of the modifications that solvent contact provokes in membranes decreasing its permeability, the impermeabilization produced by the membrane modifications is not complete and the solvent enters into the cell denaturing proteins and provoking a general stress response. This response involves, among others, the induction of several chaperones that refold proteins denatured by the solvents. In response to solvents, the GroES, Tuf-1 and CspA chaperones levels increased as deduced from proteomic studies (Segura *et al.*, 2005) and the regulon is activated by σ^{32} in *P. putida* (Domínguez-Cuevas *et al.*, 2006).

2.3 Solvent metabolism

Small amounts of the solvents can also be degraded by the metabolic pathways present in bacteria, using these compounds as carbon sources (Ramos *et al.*, 2002). Of the 5 different pathways known to be able to degrade toluene to catechols (TOL, TOD, TMO, TOM and TBU pathways), three of them are present in *Pseudomonas* strains (TOL, TOD and TMO). *Pseudomonas putida* DOT-T1E is able to degrade toluene to Krebs cycle intermediates via the TOD pathway (Lau *et al.*, 1997; Mosqueda *et al.*, 1999), but mutants in genes of the catabolic pathway were

as tolerant to aromatic hydrocarbons as the parental strain, suggesting that the degradation pathway is not essencial for resistance to solvents. Nonetheless, it should be taken into account that the metabolism of toluene generates energy to overcome damage induced by this aromatic compound (Segura *et al.*, 2005; Domínguez-Cuevas *et al.*, 2006).

2.4 Efflux pumps

Another efficient but energetically costly defense mechanism is the removal of the solvents by efflux pumps. Efflux pumps are considered to be the most efficient mechanisms of solvent tolerance in Gram-negative bacteria (Segura *et al.*, 2012). Efflux pumps are widely distributed in all organisms, but their specificity, affinity for solvents and rate of removal influence their role in solvent extrusion. In bacteria, the genes encoding these pumps are located on the chromosome and on plasmids (Piddock, 2006a). These systems, called MDR (*multidrug resistance*) efflux pumps, catalyze the active expulsion of antibiotics, xenobiotic and solvents from the cell (membrane, periplasm or cytoplasm) to the outer medium (Lomovskaya *et al.*, 1999; Lomovskaya *et al.*, 2001; Piddock, 2006b; Nikaido and Takatsuka, 2009). The first evidences demonstrating the implication and importance of energy-dependent processes in solvent extrusion in bacteria were provided by Isken and de Bont (1996) and Ramos *et al.* (1997) who isolated solvent-sensitive mutants in *P. putida* S12 and DOT-T1E respectively, that exhibited impaired functioning of efflux pumps. After that, numerous studies identified several efflux pumps in different bacteria; for instance, in *E. coli* K12 strain it was described that the AcrAB efflux pump, which is ultimately involved in the extrusion of antibiotics, is also relevant in solvent extrusion (White *et al.*, 1997; Aono, 1998). In *P. aeruginosa* MexAB-OprM, MexCD-OprJ and MexEF-OprN are also able to extrude solvents (Li *et al.*, 1998), and in *P. fluorescens* cLP6a an efflux pump called EmhABC was shown to be able to extrude polycyclic aromatic hydrocarbons (Bugg *et al.*, 2000; Hearn *et al.*, 2003).

3. Generalities on efflux pumps

3.1 MDR transporter systems

It is well known that, in bacteria, efflux pumps can confer decreased susceptibility to antibiotics, what is vital for its survival in competitive niches. *Pseudomonas* strains, for instance, present different MDR efflux pump systems that are critical for its antibiotic resistance profiles (Ramos *et al.*, 2002; Paulsen, 2003). Efflux pumps associated with MDR can be specific for one substrate or can transport a range of different chemicals.

MDR efflux pumps are classified based in the number of transmembrane-spanning regions that the transporter proteins present, the number of components that form the pump (single or multiple), their source of energy and the types of substrate that they can extrude (Poole, 2004; Piddock, 2006b). According to that, drug efflux systems are grouped in 5 families: the multidrug and toxic-compound extrusion (MATE) family, the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family and the resistance nodulation-cell division (RND) family (Figure 3).

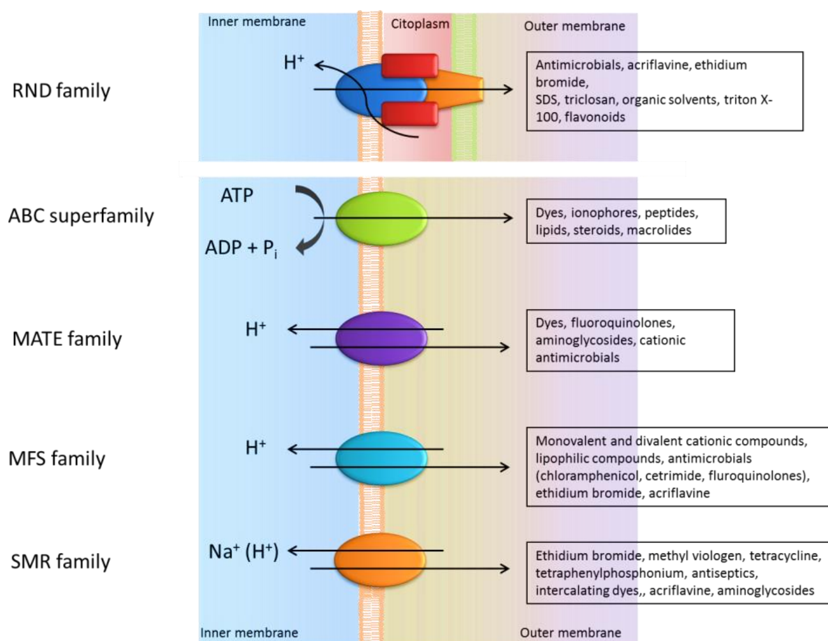

Figure 3. Bacterial drug and multidrug efflux pumps found in Gram-positive and Gram-negative bacteria. Adapted from Piddock (2006b).

3.1.1 MATE family

The MATE family consists of sodium ion-driven drug efflux pumps. Although proteins of the MATE family are not well characterized, the family was identified with the finding of NorM, a multidrug Na^{+}-antiporter from *Vibrio parahaemolyticus* that confers resistance to dyes, fluoroquinolones and aminoglycosides to this microorganism (Morita *et al.*, 1998). NorM was also found later in *Neisseria* species (Rouquette-Loughlin *et al.*, 2003) and in *E. coli* (the YdhE pump) where it confers resistance to cationic antimicrobials (Yang *et al.*, 2003).

3.1.2 ABC superfamily

One of the principal types of MDR efflux systems is ABC-type efflux transporters that remove drugs coupled to ATP hydrolysis. This MDR system represents the major mechanism of efflux pumps in eukaryotes. Although most ABC transporters were discovered as specific drug transporters, they frequently transport a wide range of substrates, including dyes, ionophores, peptides, lipids and steroids. They are characterized by the presence of a conserved sequence of approximately 215 amino acids forming an ATP binding domain (Young and Holland, 1999). Many of these transporters belong to the ABC superfamily that, in humans, include the clinically significant multidrug resistance pumps P-gp (P-glycoprotein) and MRP (multidrug resistance protein), both conferring resistance to anticancer drugs (Bellamy, 1996; Gottesman *et al.*, 1996; Leslie *et al.*, 2001). P-gp homologues have been found in bacteria such as LmrA multidrug transporter from *Lactococcus lactis*, which can functionally substitute P-gp in human lung fibroblast and exhibits a substrate specificity similar to the human protein (Poelarends *et al.*, 2002), or the MacB transporter from *E. coli* involved in the efflux of macrolide antibiotics (Kobayashi *et al.*, 2001).

3.1.3 MFS family

MFS family is a group found in bacteria, archaea and eukaryotes. It is composed by secondary transporters formed by a 400 amino acids polypeptide that are driven by chemiosmotic energy and includes proton/drug antitransporters (Pao *et al.*, 1998; Saier *et al.*, 1999). QacA in *S. aureus* and Mbr in *Bacillus subtilis* (Neyfakh, 1992; Brown and Skurray, 2001) are the MFS efflux pumps best characterized. A number of highly conserved amino acid motifs have been identified within MFS proteins which are likely to be essential for the structure

and/or function of these transporters (Paulsen *et al.*, 1996). In the case of QacA, this efflux pump provides resistance to monovalent and divalent cationic, lipophilic or antimicrobial compounds via a proton motive force-dependent antiport mechanism. The *qacA* gene is borne by a multidrug resistance plasmid from a clinical isolate of *S. aureus*. MFS family shows high relevance at a clinical level, being critical efflux pump systems of Gram-positive bacteria related with clinical infections (Piddock, 2006b). Other examples of MFS efflux pumps with clinical relevance present in *S. aureus* is NorA, which confers resistance to chloramphenicol and fluoroquinolones, as well as to dyes and biocides, such as cetrimide (Neyfakh, 1992; Neyfakh *et al.*, 1993), or in *S. pneumoniae*, where PmrA efflux pump exports the fluoroquinolones ciprofloxacin and norfloxacin, as well as the dyes acriflavine and ethidium bromide (Gill *et al.*, 1999).

3.1.4 SMR family

The SMR family is a proton-driven drug efflux pump only found in prokaryotes. They are much smaller than transporters belonging to other families like RND. SMR pumps are normally composed of around 100 amino acids that are putatively arranged into four helices (Paulsen *et al.*, 1996). Gram-negative multidrug efflux pumps appear to catalyze extrusion across the cytoplasmic membrane. The best characterized SMR pump is EmrE from *E. coli*, a multidrug transporter that contributes to resistance to ethidium bromide and methyl viologen, tetracycline, and tetraphenylphosphonium, as well as other antiseptics and intercalating dyes (Yerushalmi *et al.*, 1995; 1996). In *P. aeruginosa* it was found another SMR pump with close identity to EmrE that plays an important role in the intrinsic resistance of the strain to ethidium bromide, acriflavine and aminoglycoside antibiotics (Li *et al.*, 2003).

3.1.5 RND family

Efflux pumps of the RND family are found only in prokaryotes and are associated with clinically significant resistance in Gram-negative bacteria where are considered the most efficient proteins mediating the active efflux of many antibiotics, chemotherapeutic agents and solvents. These pumps are proton-driven systems that normally become associated with other two classes of proteins, the outer membrane channel, such as TolC of *E. coli* and OprM of *P. aeruginosa*, and the periplasmic “adapter” protein, such as AcrA of *E. coli* and MexA of *P.*

aeruginosa, which together form a multicomponent complex extending from the inner membrane to the outer membrane (Nikaido and Takatsuka, 2009). This molecular organization permits bacteria to expulse compounds from the periplasm or from the cytoplasm to the external medium. This kind of transporters are composed typically of approx. 1000 amino acid residues adopting a 12-helical structure (Borges-Walmsley *et al.*, 2003).

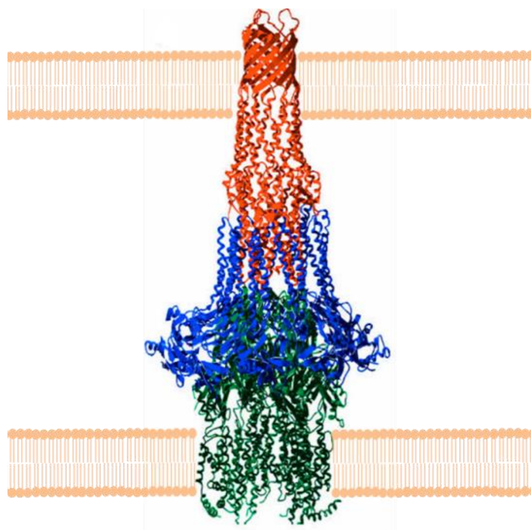

Figure 4. Crystal structure of TolC, AcrA, and AcrB. Hypothetical arrangement of the tripartite TolC (red)–AcrA (blue)–AcrB (green) structure based on X-ray crystal structures. Adapted from Nikaido and Takatsuka (2009).

Most of Gram-negative bacteria encode for a large number of RND pumps in their genomes. The best studied members of this efflux system are represented by AcrAB/TolC from *E. coli* (Koronakis *et al.*, 2000; Murakami *et al.*, 2002; Tamura *et al.*, 2005; Lobedanz *et al.*, 2007) and OprM-MexA-MexB efflux system from *P. aeruginosa* (Akama *et al.*, 2004; Higgins *et al.*, 2004). Indeed, *P. aeruginosa* encodes for other RND pumps such as MexXY-OprM, MexCD-OprJ and MexEF-OprN, which altogether act synergically conferring resistant to several antimicrobial compounds, acriflavine, ethidium bromide, SDS, triclosan and some organic solvents (Poole and Srikumar, 2001; Piddock, 2006b). The system described in *E. coli* is also found in *S. typhimurium* and *S. enteriditis*, including as substrates several antibiotics, acriflavine, ethidium bromide, bile salts, SDS, Triton X-100, cetrimide and triclosan (Baucheron *et al.*, 2004; Eaves *et al.*, 2004). Other clinical bacteria as *C. jejuni* and *N. gonorrhoeae* also encodes for RND efflux pumps such as CmeABC and MtrCDE, respectively, which confer antibiotic resistance, just highlighting the critical role of RND pumps in clinical isolates.

Crystallization of the complete tripartite structure allowed a better understanding of the mechanism of RND pumps (Figure 4) and permitted to observe that they form a continuous/undisrupted channel between the inner and outer membranes, which allow the substrates to be expelled to the outer medium (Murakami, 2008; Nikaido and Takatsuka, 2009).

3.2 RND efflux pumps in *Pseudomonas putida* DOT-T1E

The genome of *P. putida* DOT-T1E encodes for up to 20 RND efflux pumps (Godoy *et al.*, 2010; Udaondo *et al.*, 2012), which can be relevant in the tolerance against noxious compounds. Segura *et al.* (2003) carried out southern hybridization analysis where the authors found that highly resistant strains such as *P. putida* DOT-T1E or *P. putida* MTB6 presented three relevant efflux pumps involved in the extrusion of solvents and antibiotics. These three efflux pumps have been identified as the main elements related with solvent tolerance (Rojas *et al.*, 2001). Survival analysis of DOT-T1E cultures after a shock with toluene revealed that these three RND efflux pumps, with different but overlapping substrate specificity, are directly involved in toluene resistance since a triple mutant in the three RND efflux pumps is hypersensitive to toluene (Rojas *et al.*, 2001; Segura *et al.*, 2012; Udaondo *et al.*, 2013).

These three RND efflux pumps are called TtgABC (Fukumori *et al.*, 1998; Ramos *et al.*, 1998), TtgDEF (Mosqueda and Ramos, 2000) and TtgGHI (Rojas *et al.*, 2001) (*toluene tolerance gene*). The *ttgABC* and *ttgDEF* operons are borne on the chromosome while *ttgGHI* operon is located in the pGRT1 plasmid (Rodríguez-Herva *et al.*, 2007). TtgABC is widely distributed in *P. putida* strains, just emphasizing the importance of the role of this efflux pump within the solvent-tolerance population. TtgDEF is present only in strains which carry the *tod* pathway for toluene degradation (Mosqueda and Ramos, 2000; Phoenix *et al.*, 2003). The pGRT1 plasmid encoding TtgGHI pump presents a high stability and is self-transmissible to other *P. putida* strains, thus constituting a valuable tool for biotechnological research.

The complex regulation of the three efflux pumps suggests that their coordinated expression confers the high level of resistance displayed by DOT-T1E strain. Although the three Ttg efflux pumps are able to extrude flavonoids and a wide range of antibiotics, the TtgABC pump is the most important element related with biotic extrusion, being able to extrude ampicillin, chloramphenicol,

tetracycline and flavonoids in addition to toluene and other solvents (Terán *et al.*, 2003; Guazzaroni *et al.*, 2005; Terán *et al.*, 2006; Duque *et al.*, 2007). TtgDEF has been shown to be involved in aromatic hydrocarbon detoxification (Mosqueda and Ramos, 2000). The TtgGHI pump is considered as a key factor in high level toluene resistance due to its main role in solvent removal (Segura *et al.*, 2003), but also plays a secondary role in antibiotic extrusion.

4. RND efflux pumps regulation

Since RND pumps are the main players in solvent tolerance, we examine in this section some of the aspects of what it is known about their mechanism of regulation at the transcriptional level. Multidrug efflux pumps are constitutively expressed at a low basal level, but the high energy costs that provoke the production of these proteins suggested that the MDR synthesis would need a regulatory system to control their expression levels (Lee and Edlin, 1985; Alonso *et al.*, 2004). Moreover, excessive expression of these pumps could have a negative effect in bacteria, due to a physical disruption of membrane integrity or to an unwanted export of essential metabolites. Also, an increase in the presence of transporter substrates or certain physiological or environmental conditions provoke an increase in the expression of these systems, being regulated by different mechanisms and at many regulatory levels, just reflecting the different cellular functions in which they can be involved (Grkovic *et al.*, 2002).

The most recurrent DNA-binding motif for the binding of regulators to their corresponding promoters is a conserved DNA recognition motif that consists of a α-helix, a turn, and a second α-helix (HTH motif). The latter helix is known to be the recognition helix involved in the direct contact with the DNA target (Pabo and Sauer, 1992; Krell *et al.*, 2007), generally in the major groove of the DNA structure. In general, HTH proteins bind as dimers to symmetrical DNA sequences in which each monomer recognizes a half-site (Harrison and Aggarwal, 1990), although variations have been found and some proteins recognize direct repeats (Grainger *et al.*, 2004). In relation with 3D structural motifs, conserved domains and primary sequences, HTH transcriptional regulators have been grouped in at least 17 different families such as: MerR family (Molina-Henares *et al.*, 2010), MarR family (Alekshun *et al.*, 2001), TetR family (Ramos *et al.*, 2005), LysR family (Schell, 1993), AraC family (Tobes and Ramos, 2002), or IclR family (Molina-Henares *et al.*, 2006).

According to the different regulatory levels, efflux pumps are generally subject to regulation at a local level, where we can find examples of transcriptional repression and activation by proteins encoded adjacent to that for the transporter (Grkovic *et al.*, 2001); but some of these transporters have also been identified to be regulated by global regulatory systems.

4.1 Efflux pumps local regulation

Almost all encoded genes related with MDR pumps are controlled at the transcriptional level by local activators or repressors, usually located adjacent to the transporter of the MDR. The best characterized are TetR, a repressor of *tetA* gene, transporter of tetracyclines; the activator BmrR of *bmr* gene in *B. subtilis* (Ahmed *et al.*, 1994); QacR in *S. aureus*, that regulates the transporter gene *qacA* (Grkovic *et al.*, 1998) or TtgV, an IclR-family member that is the main modulator in the regulation of the TtgGHI efflux pump in *P. putida* DOT-T1E (Guazzaroni *et al.*, 2005; Guazzaroni *et al.*, 2007; Fillet *et al.*, 2009).

4.1.1 The TetR family regulators

QacR, CprB or EthR are some of the more than 3000 non-redundant regulatory proteins that belong to the TetR family of repressors (Ramos *et al.*, 2005). These are characterized by 47 amino acid residues that form the HTH DNA binding motif and adjacent regions in their three-dimensional structures. This family is named TetR because of the member of the group that has been most completely characterized genetically and biochemically, the TetR protein (Figure 5) (Hillen and Berens, 1994; Helbl *et al.*, 1995; Bertram and Hillen, 2008). This protein is a homodimer that controls the expression of the *tet* genes, whose products confer resistance to tetracycline (Hinrichs *et al.*, 1994). TetR binds to two identical operators separated by 11 bp and located in the intergenic region between the *tet*R and *tetA* genes, thus preventing transcription from both promoters. The operator sequences overlap with promoters of *tetA* and *tet*R, thereby blocking the expression of both genes, but when tetracycline complexes with Mg^{2+} bound to TetR, a conformational change takes place and avoid the TetR-DNA binding (Jogun and Stezowski, 1976).

The TetR is constituted by two identical monomers that fold into 10 α-helices with connecting turns and loops. The structure of the TetR monomer is stabilized mainly by hydrophobic helix-to-helix contacts and can be divided into two

domains, a DNA-binding domain at the N-terminal end, and a regulatory core domain involved in dimerization and ligand binding (Hinrichs *et al.*, 1994; Ramos *et al.*, 2005). The DNA-binding domain is constituted by helices α1, α2, α3 and their symmetrics α1', α2' and α3' while α4 and α4' connect these domains with the regulatory core domain composed of helices α5 to α10 and their symmetrics.

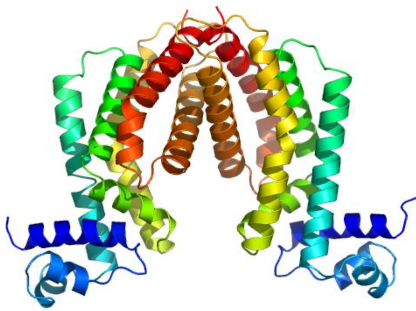

Figure 5. Cystal structure of a TetR homodimer of *Thermotoga maritima*. (See for more details Joint Center for Structural Genomics, accession TM1030).

Other well characterized regulator of TetR family, is QacR, another repressor that regulates the expression of QacA MDR efflux pump system in *S. aureus* (Aramaki *et al.*, 1995). The *qac* locus is located on a plasmid which can be transferred to other bacteria conferring resistance to monovalent or bivalent cationic lipophilic antiseptics and disinfectants such as quaternary ammonium compounds (Qac). QacR represses QacA transcription by blocking the RNA polymerase binding site at the QacA promoter region. It is able to bind a wide range of cationic lipophilic drugs, such as rhodamine 6G, crystal violet or ethidium (Schumacher *et al.*, 2001; 2002).

4.1.2 The IclR family regulators

IclR transcriptional regulator name to a whole family of transcriptional regulators present in bacteria and archaea. This founding member regulates the *aceBAK* operon of *E. coli*, which encodes enzymes involved in the glyoxylate bypass, what lets the bacteria to grow on acetate as a sole carbon source (Kumari *et al.*, 2000). Proteins of this family regulate diverse functions such as the glyoxilate cycle in enterobacteriaceae (Yamamoto and Ishihama, 2003), degradation of aromatics by soil bacteria (Gerischer *et al.*, 1998), inactivation of quorum sensing signals in *Agrobacterium* (Zhang *et al.*, 2004), plant virulence by certain enterobacteriaceae (Reverchon *et al.*, 1991), sporulation in *Streptomyces* (Jiang and

Kendrick, 2000) and resistance to toxic compounds in *Pseudomonas* (Mosqueda and Ramos, 2000; Rojas *et al.*, 2003; Guazzaroni *et al.*, 2004).

Members of this family include repressors such as PcaR of *P. putida* and CatR of *Rhodococcus opacus* 1CP (Eulberg and Schlomann, 1998; Gerischer *et al.*, 1998), activators (PobR of *Acinetobacter sp.,* TsaQ of *Comamonas testosteroni* and YiaJ of *E. coli*) (DiMarco *et al.*, 1993; Schumacher *et al.*, 2002; Tralau *et al.*, 2003) and proteins with a dual role acting as a repressor for certain genes and as activator for others such as PcaU of *Acinetobacter* sp. and GenR of *Corynebacterium glutamicum* (Trautwein and Gerischer, 2001; Chao and Zhou, 2013). In general, IclR regulator genes lie upstream of its target gene cluster and are transcribed in the opposite direction (Tropel and van der Meer, 2004). Proteins of the IclR family always have the HTH motif at their N-terminus end (Donald *et al.*, 1996; Pan *et al.*, 1996) and bind target promoters as dimers or as a dimer of dimers. The polypeptide size of these members is typically in the range of 240-280 residues.

The resolution of the structure of IclR of *Thermotoga maritima* (Zhang *et al.*, 2004) permitted to define some of the structural characteristic of the IclR family members. The N- and C-terminal domains are connected by a linker helix, which participates in protein dimerization through its N-terminal side. In the case of IclR of *T. maritima*, the protein conformation is a tetramer but different oligomeric states of the IclR family like homodimers for the regulator PcaU (Popp *et al.*, 2002) have been described (Figure 6). The two subunits within one IclR dimer interact solely at the interface of their DNA binding domains, what results in a relatively short palindromic DNA sequence with specific contacts in the major groove of the DNA that is favorable for binding.

On the other hand, the C-terminal domains are involved in subunit multimerization and effector binding. The C-terminal crystal structure of AllR, a member of IclR family, helped to identify the effector recognition site, highlighting the importance of some amino acids forming a hydrophobic patch in the effector binding site (Walker *et al.*, 2006).

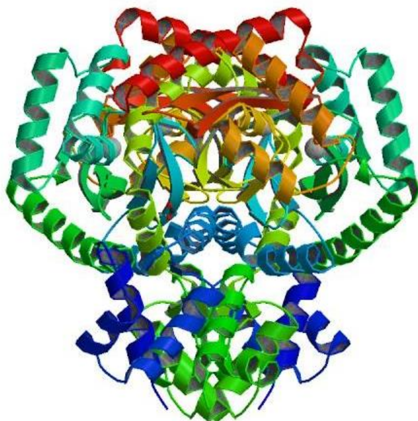

Figure 6. IclR dimer arrangement as derived from the crystal structure. Dimer view showing that the interface is formed exclusively between the two HTH DNA binding domains. (See for more details PDB accession. 1MKM).

4.2 Efflux pumps global regulation

Although most of transporter genes are regulated at a local level, there are many examples of global transcriptional regulators like for example AcrAB regulation in *E. coli* and MexAB-OprM in *P. aeruginosa*. One of the best studied cases is the regulation of AcrAB efflux pump. This pump is locally regulated by AcrR but there are global regulators such as MarA, Rob and SoxS (Alekshun and Levy, 1997; Pomposiello and Demple, 2000), that also regulate the *acrAB* operon, being an important factor in the *mar* (*multiple antibiotic resistance*) phenotype and in the phenotype of resistance to solvents. These three global regulators with the Fis modulator (Martin and Rosner, 1997) regulate the response of AcrAB transporter to deleterious compounds for cells and adverse environmental and growth conditions.

AcrAB is also positively regulated by SdiA, a protein associated to quorum sensing which expression depends on the growth phase (Rand *et al.*, 2002), and regulates genes involved in cellular division (Rahmati *et al.*, 2002), what suggests a role of this efflux pump in the extrusion of quorum sensing signals together with a wide variety of toxic compounds.

4.3 Regulation of TtgABC, TtgDEF and TtgGHI efflux pumps in *Pseudomonas putida*

In the last years, extensive analysis of the regulation of the *ttgABC*, *ttgDEF* and *ttgGHI* operons of *P. putida* KT2440 and DOT-T1E have been carried out. TtgABC is expressed at a relatively high basal level (Ramos *et al.*, 1998), increasing its expression in the presence of certain antibiotics and flavonoids in the culture medium (Duque *et al.*, 2001; Terán *et al.*, 2003; Terán *et al.*, 2006). On the other hand, TtgGHI efflux pump also has a basal level of expression, which increases in the presence of solvents (Duque *et al.*, 2001). TtgDEF is not expressed at a basal level but is induced by solvents (Mosqueda and Ramos, 2000). Genes belonging to the three efflux pumps are grouped in operons, and divergently from the efflux pump operons is present a gene that encode the regulatory protein of each system (Figure 7).

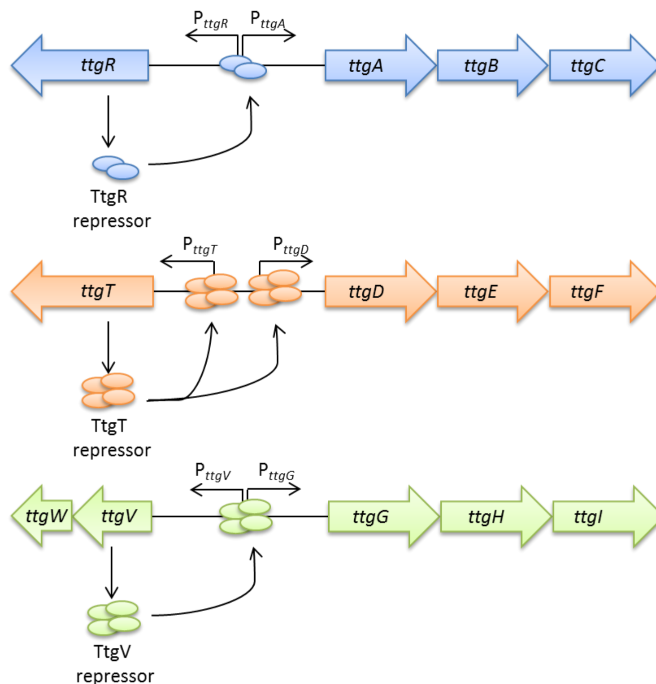

Figure 7. Genetic organization of TtgABC, TtgGHI and TtgDEF efflux pumps in *P. putida* DOT-T1E.

TtgR, the regulator of TtgABC efflx pump, is encoded divergently to the *ttgABC* operon. This transcriptional regulator belongs to the TetR family of

transcriptional regulators (Ramos *et al*., 2005) and modulates the transcription of the pump genes (Terán *et al*., 2003; Terán *et al*., 2006). TtgR is able to bind to a wide range of compounds. Its crystal structure resolution has increased the understanding of its regulatory mechanism (Alguel *et al*., 2007; Krell *et al*., 2007; Daniels *et al*., 2010). The expression of *ttgDEF* and *ttgGHI* operons is controlled by TtgT and TtgV, respectively, both of them belonging to the IclR family of transcriptional regulators (Rojas *et al*., 2003; Terán *et al*., 2007). TtgGHI and TtgDEF show a more complex regulation because of the capacity of TtgT and TtgV to bind the same promoter regions. It is important to notice that TtgR and TtgV also regulate their own expression via auto-control, what permit cells to respond quickly to a solvent stress, saving an important amount of energy in the cell.

4.4 The TtgR transcriptional regulator

TtgR is a protein of 210 amino acids that binds to a pseudo-palindromic sequence extending over 28 bp located in the *ttg*R/*ttgA* intergenic region and overlaps the -10 and -35 regions of *ttgABC* promoter, and the -10 region of the *ttg*R promoter (Krell *et al*., 2007). TtgR is a dimeric protein that binds to its target operator in a similar way to that of QacR, a dimer which binds to opposit faces of the operator, and the monomers belonging to different dimers bind to each half of a palindrome (Schumacher *et al*., 2002). In the absence of effectors, the TtgR dimer is bound to its operator site repressing its own expression and that of the efflux pump. The binding of an effector to the protein-DNA complex induces the dissociation of TtgR, and allows transcription (Alguel *et al*., 2007). TtgR crystallization was elucidated and it was shown that a TtgR monomer consists of nine α-helices forming two distinct domains. The DNA binding domain consists of α4-α9 with an angle of approximately 80º between the two domains. The ligand binding domain showed a hydrophobic binding pocket, which explains TtgR's ability to bind different ligands (Alguel *et al*., 2007; Segura *et al*., 2012). Within this pocket two binding sites were identified, one that binds ligands with high affinity, and the second which binds molecules with low affinity, both separates by the chemical properties of ligands, such as stereochemistry, conformation and size. The only common feature of the ligands is the presence of at least one aromatic ring in their structures.

4.5 The TtgV transcriptional regulator

TtgV is a protein of 259 amino acids which belongs to the IclR family of transcriptional regulators and is the main regulator in the modulation of the expression of *ttgDEF* and *ttgGHI* (Fillet *et al.*, 2012). As the most commonly used mechanisms in repression systems (Rojo, 2001; Yamamoto and Ishihama, 2003; Molina-Henares *et al.*, 2006), TtgV-DNA binding prevents RNA polymerase access to the promoter region through physical competition and by introducing a bend that impedes RNA polymerase contact (Guazzaroni *et al.*, 2004).

TtgV is a tetramer in solution and binds as a tetramer to its target DNA operators. Guazzaroni *et al.* (2007) demonstrated that TtgV binds a 42 bp target operator in the *ttgDEF* and *ttgGHI* intergenic regions covering the -10 region of each promoter. Footprint assays revealed that when TtgV is bound to *ttgGHI* operator, it bends the DNA (Rojas *et al.*, 2003; Guazzaroni *et al.*, 2004). Atomic force microscopy (AFM) assays defined DNA torsion of 57º when TtgV was bound to the *ttgV-ttgG* intergenic region, and 40º as a result of TtgV binding to the *ttgD* promoter (Guazzaroni *et al.*, 2007).

It has been shown that the TtgV protein binds an extensive range of mono and bi-aromatic compounds such as toluene, xylenes, benzonitrile, indole and naphtalenes (Guazzaroni *et al.*, 2004; Fillet *et al.*, 2009). This provokes a conformational change in TtgV, which leads to its release from the target DNA, thus permitting the RNA polymerase to bind the *ttgG-ttgV* promoter region and finally initiate *ttgG* and *ttgV* transcription (Fillet *et al.*, 2012).

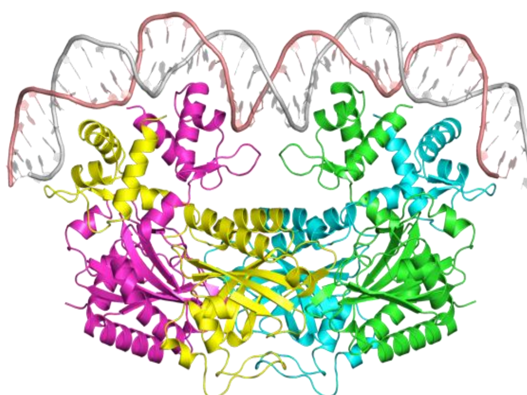

Figure 8. TtgV/DNA complex. Representation of the interaction of each TtgV monomer with its target DNA at the *ttgG* operator. Each monomer is represented in a different color (Fillet *et al.*, 2012).

The 3D structure of TtgV revealed that it is composed of an N-terminal domain which is able to bind DNA, and a C-terminal effector-binding domain, both linked via a long α-helix. The inspection of the co-crystal structure showed that R47, T49, R52 and L57, in the recognition helix, are involved in the direct contact to DNA, results that were confirmed by site-directed mutagenesis (Fillet *et al.*, 2009; Lu *et al.*, 2010). In addition, S35, located outside the HTH domain was also found to be involved in establishing a contact with DNA (Lu *et al.*, 2010). Mutants generated in the region corresponding to the effector-binding domain showed an abolition of effector binding, what is consistent with a model by which effector binding initiates an intramolecular signal transmission that impacts on the linker region, thus causing conformational changes leading to protein dissociation from DNA (Guazzaroni *et al.*, 2007; Fillet *et al.*, 2012).

Comparison of the apo-TtgV structure with the structure of the TtgV-DNA complex (Figure 8) showed a major re-arrangement in TtgV. In the DNA-bound structure, the DNA-binding domain was moved out by an angle of almost 90º, which was possible by the introduction of a very pronounced kink at residue Q86 in the linker helix (Lu *et al.*, 2010).

Differential scanning calorimetry (DSC) assays revealed that TtgV unfolds in a single event, thus suggesting that there is functional communication between both domains leading to cooperative unfolding. Mutants in residues R98 and E102 in the linker helix showed an unfolding in two events, indicating consecutive unfolding of both domains (Fillet *et al.*, 2011). Moreover, the E102R mutant was deficient in release from the DNA promoter in the presence of the effectors. In this line, V223A and Q51A mutants also showed a role in the TtgV signaling pathway between the effector binding and the DNA-binding domains (Fillet *et al.*, 2009). This data show that the cross-talk involves amino acid residues that are far situated neither in the primary amino acid sequence nor in the 3D structure. In that sense, our group explored some aspects still unknown in TtgV protein and its role in the regulation of TtgGHI efflux pump in antibiotic resistance of *P. putida* DOT-T1E.

5. Antibiotics

5.1 Generalities and discovery

As have been mentioned above, efflux pumps are the main mechanisms used by bacteria to develop resistance to antibiotics, as they do for developing resistance to biocide compounds and solvents. Hence, there is a relationship between the mechanisms that bacteria use to defend against organic solvents and antibiotics. The presence of solvents in the environment and the indiscriminate use of biocides promote the development of resistance to antimicrobials and, in particular, cross-resistance to antibiotics (Daniels and Ramos, 2009). This phenomenon has been well demonstrated in *E. coli* (Ishikawa *et al.*, 2002) which acquired multidrug resistance when bacteria was treated with low doses of cetyltrimethylammonium bromide (CTAB), thus highlighting that it is possible that multidrug resistant bacteria occur anywhere in industrial, medical and domestic environments.

Antibiotics can be defined as natural (usually produced by bacteria or fungi), synthetic or semisynthetic molecules that are able to provoke death or stop the growth of bacteria, viruses and fungi. Antimicrobial compounds form a very heterogeneous group of substances which show different pharmacokinetic and pharmacodynamic behaviors, acting as "blockers" of structures or microbial cell processes. They show a high biological strength even at low concentrations and they present selective toxicity.

In antibiotics history, two main discoveries were particularly important; one of them was the discovery of penicillin by Alexander Fleming in 1929 (Fleming, 2001) and, after that, Florey and Chain demonstrated the possibility to extract the compound from supernatants of *Penicillium notatum* and to use them for therapeutics (Chain *et al.*, 1993). The other one was the discovery in 1935 of the curative effects of Prontosil in streptococcus infections, which was the precursor of sulfonamides. The application of penicillin as a therapeutic agent in 1942 opened a new era in antimicrobial chemotherapy (Pathania and Brown, 2008). After that, many additional classes of antibiotics spanning a broad range of chemical structures and targets soon followed, forming the foundation of the current armory of antibiotics. All of these agents are efficacious because they are able to inhibit processes that are essential for bacterial growth (Smith and Romesberg, 2007).

5.2 Families and mode of action of antimicrobials

Antibiotics can be classified in different ways depending on the cellular target, in addition to whether they induce cell death (bactericidal drugs) or inhibit cell growth (bacteriostatic drugs). Most current antimicrobials inhibit DNA, RNA, cell wall or protein synthesis (Walsh, 2003; Kohanski *et al.*, 2010) processes (Table 1).

Quinolones family, which are derivatives of nalidixic acid, act by targeting topoisomerase II (primary target in Gram-negative bacteria) and topoisomerase IV (primary target in Gram-positive bacteria), trapping these enzymes at the DNA cleavage stage and preventing strand rejoining (Critchlow and Maxwell, 1996; Kampranis and Maxwell, 1998; Heddle and Maxwell, 2002). The net effect of quinolones is to generate double-stranded DNA breaks that covalently (but reversibly) link topoisomerases (Figure 9), thus leading to inhibition of DNA synthesis, which immediately leads to bacteriostasis and eventually cell death (Kohanski *et al.*, 2010).

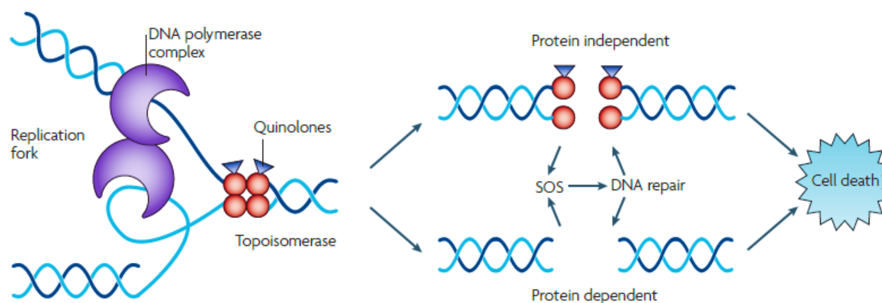

Figure 9. Quinolone mode of action (Kohanski *et al.,* 2010).

Inhibition of cell wall synthesis is provoked by β-lactams and glycopeptides. In that case, antimicrobials interfere with specific steps in cell wall biosynthesis what provoke changes in cell shape and size, induction of cell stress responses and ultimately cell lysis (Tomasz, 1979). β-lactams (penicillins, carbapenems and cephalosporins) act specifically blocking the crosslinking of peptidoglycan units by inhibiting the peptide bond formation reaction catalyzed by penicillin binding proteins (PBPs) (Tipper and Strominger, 1965; Wise and Park, 1965). By contrast, glycopeptide antibiotics (vancomycin), inhibit peptidoglycan synthesis by binding peptidoglycan units and by blocking transglycosylase and PBP activity (Kahne *et al.*, 2005).

Table 1. Antibiotic families, targets and pathways (modified from Kohanski *et al.,* 2010).

Drug type	Drug	Source	Species range	Primary target	Pathways affected
Fluoroquinolones					
DNA synthesis inhibitors	Nalidixic acid, ciprofloxacin, levofloxacin and gemifloxacin	Synthetic	Aerobic Gram-positive and Gram-negative species	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA-replication, SOS response, cell division, ATP generation, TCA cycle, Fe-S cluster synthesis, ROS formation
Trimethoprim-sulfamethoxazole					
DNA synthesis inhibitors	Co-trimoxazole (combination of trimethoprim and sulfamethoxazole in a 1:5 ratio)	Synthetic	Aerobic Gram-positive and Gram-negative species, some anaerobic Gram-negatives	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Rifamycins					
RNA syntesis inhibitors	Rifamycins, rifampin and rifapentine	Natural and semi-synthetic forms of ansamycins	Gram-positive and Gram-negative species, and *M. tuberculosis*	DNA-dependent RNA polymerase	RNA transcription, DNA replication and SOS response
β-lactams					
Cell wall synthesis inhibitors	Penicillins (penicillin, ampicillin), cephalosporins and carbapenem (imipenem)	Natural and semi-synthetic forms of carbonyl lactam ring-containing azetidinone molecules	Aerobic and anaerobic Gram-positive and Gram-negative species	Penicillin-binding proteins	Cell wall synthesis, cell division, autolysin activity, SOS response, TCA cycle, Fe-S cluster synthesis, ROS formation, envelope and redox-responsive two-component systems

Glycopeptides and glycolipopeptides					
Cell wall synthesis inhibitors	Vancomycin, teicoplanin	Natural and semi-synthetic forms of amino sugar-linked peptide chains (for glycopeptides) or of fatty acid-bearing, amino sugar-linked peptide chains (for glycolipopeptides)	Gram-positive species	Peptidoglycan units (terminal D-Ala-D-Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation and autolysin activation
Lipopeptides					
Cell wall synthesis inhibitors	Daptomycin and polymixin B	Natural and semi-synthetic forms of fatty acid-linked peptide chains	Gram-positive species (daptomycin), Gram-negative species (polymixins)	Cell membrane	Cell wall synthesis and envelope two-component systems
Aminoglycosides					
Protein synthesis inhibitors	Gentamicin, tobramycin, streptomycin and kanamycin	Natural and semi-synthetic forms of amino sugars	Aerobic Gram-positive and Gram-negative species and *M. tuberculosis*	30S ribosome	Protein translation, SOS response, TCA cycle, Fe-S cluster synthesis, ROS formation
Tetracyclines					
Protein synthesis inhibitors	Tetracycline and doxycycline	Natural and semi-synthetic forms of four-ringed polyketides	Aerobic Gram-positive and Gram-negative species	30S ribosome	Protein translation (through inhibition of aminoacyl tRNA binding to ribosome)

Macrolides					
Protein synthesis inhibitors	Erythromycin and azythromycin	Natural and semi-synthetic forms of 14- and 16-membered lactone rings	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion
Streptogramins					
Protein synthesis inhibitors	Pristinamycin, dalfopristin and quinupristin	Natural and semi-synthetic forms of pristinamycin I and II	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of initiation, elongation and translocation steps) and free tRNA depletion
Phenicols					
Protein synthesis inhibitors	Chloramphenicol	Natural and semi-synthetic forms of dichloroacetic acid with an aromatic nucleus and aminopropanediol chain	Some Gram-positive and Gram-negative species, including *B. fragilis, N. meningitidis, H. influenzae* and *S. pneumoniae*	50S ribosome	Protein translation (through inhibition of elongation step)

Rifamycins, firstly isolated from *Amycolatopsis mediterranei*, are a class of semi-synthetic bactericidal antibiotics that act inhibiting RNA synthesis. These molecules inhibit DNA-dependent transcription by its binding with high affinity to the DNA dependent RNA polymerase (Losick and Pero, 1976). Rifamycins block RNA synthesis, thus being attributed to this drug the ability to sterically inhibit nascent RNA strand initialization (Campbell *et al.*, 2001; Naryshkina *et al.*, 2001; Kohanski *et al.*, 2010).

Many antimicrobial groups such as macrolides (erythromycin), lincosamides (clindamycin), streptogramins (dalfopristin-quinupristin), amphenicols (chloramphenicol) and oxazolidinones (linezolid) act by inhibiting 50S ribosome (Patel *et al.*, 2001; Katz and Ashley, 2005). In this case, these antimicrobials block either initiation of protein translation or translocation of peptidyl tRNAs, which inhibit the peptidyltransferase reaction that elongates the nascent peptide chain and eventually trigger dissociation of the peptidyl tRNA (Menninger and Otto, 1982; Vannuffel and Cocito, 1996; Kohanski *et al.*, 2010).

Tetracyclines and aminocyclitols work by inhibiting the 30S subunit of the ribosome. Tetracycline, for instance, block the access of aminoacyl tRNAs to the ribosome, while aminocyclitols, such as spectinomycin, and aminoglycosides (streptomycin, kanamycin and gentamicin) bind to the 16S rRNA component of the 30S ribosome subunit (Hancock, 1981; Davis, 1987). Spectinomycin interferes with the stability of peptidyl-tRNA binding to the ribosome by inhibiting elongation factor-catalyzed translocation, but does not cause protein mistranslation. By contrast, aminoglycosides provoke an alteration in the conformation of the complex formed between an mRNA codon and its cognate charged aminoacyl-tRNA at the ribosome, promoting tRNA mismatching which can result in protein mistranslation. (Figure 10) (Davies *et al.*, 1965; Pape *et al.*, 2000).

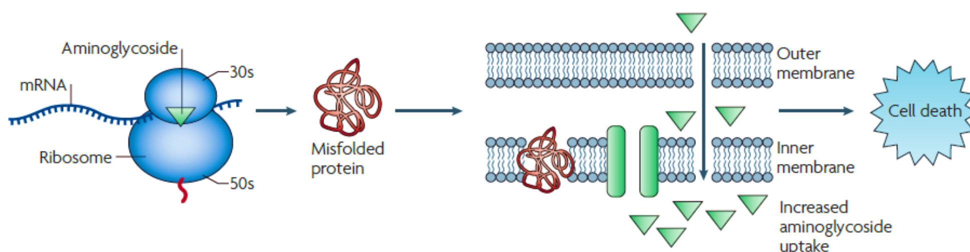

Figure 10. Aminoglycoside mode of action (Kohanski *et al.,* 2010).

To understand the mode of action of antimicrobials and how bacteria protect themselves against those compounds is basic to combat the continuous evolution of multidrug resistance bacteria and to be able to defend against them.

5.3 Multidrug resistance

In spite of the high number of antimicrobials discovered and produced every year to combat bacterial infections, multidrug resistant bacteria are emerging due to a combination of microbial characteristics such as the selective pressure of antimicrobial use what provokes mutation in target enzymes, the enzymatic inactivation of the drug (phosphorylation, acetylation, adenylation or hydrolysis), the inactivation of efflux pumps that expel antimicrobials and the acquisition of genes from other species (Dzidic and Bedekovic, 2003; Nikaido, 2009; Nikaido and Pages, 2012). This acquisition of genes, each coding for resistance to a single drug, is produced by resistance plasmids where the resistance genes are assembled by transposons and integrons (Hall and Stokes, 1993). Many of these genes apparently have their evolutionary origins in the antibiotic-producing microbes, which must defend themselves from the antibiotics they produce.

All of these mechanisms have culminated in an unprecedented acceleration in the emergence of resistance to approved antibiotics, provoking an increase in the number of difficult to treat multidrug resistant bacteria (Lipsitch and Samore, 2002; Rice, 2006; Wright *et al.*, 2006; Smith and Romesberg, 2007). That is the case of *Staphylococcus aureus* (MRSA) and Gram-negative strains such as *P. aeruginosa* and *Acinetobacter baumanii*, "pan-resistant" strains in which an outer membrane barrier of low permeability and an array of efficient multidrug efflux pumps are combined with multitude of specific resistance mechanisms (Nikaido, 2009).

To efficiently combat multidrug resistant bacteria it is necessary to understand the molecular basis of the efflux mechanism that is involved in the limitation of the intracellular (or periplasmic) concentration of all clinically used groups of antibiotics.

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II. AIM OF THE THESIS AND SPECIFIC OBJECTIVES

The function and the regulatory mechanisms of Ttg efflux pumps have been extensively studied in Ramos' group in the last few years. The characterization and physiological role of the TtgV regulator, together with the establishment of its 3D structure have led to an enhanced understanding of its role in *P. putida* tolerance.

The current work is focused on the study of the role of the TtgGHI efflux pump of *P. putida* DOT-T1E in antibiotic resistance and its effect in cross-resistance between antimicrobials and solvents. Studies involving the use of DOT-T1E efflux pump mutants have provided a deeper knowledge of their regulation and role in resistance mechanisms, leading us to explore the biotechnological use of *P. putida* DOT-T1E strains in the discovery of new antimicrobial compounds. The specific objectives of this thesis are:

- To deepen our understanding of intramolecular signal transmission in TtgV through the generation of mutants in the TtgV interlinker.
- To reveal the role of specific amino acid residues outside of the canonical HTH domain in binding of TtgV to its cognate promoters.
- To study the role of indole in *P. putida* DOT-T1E antibiotic resistance and its effect as an interspecies signaling molecule.
- To use RNA-seq to understand the response of *P. putida* to antibiotics and to identify new sRNAs from the *P. putida* chromosome.
- To develop biotechnological applications of a *P. putida* DOT-T1E mutant that is defective in antibiotic efflux pumps, with the aim of discovering new antimicrobial compounds.
- To characterize and sequence the genome of a new *Pseudomonas* strain that is capable of producing new cyclic lipodepsipeptides.
- To study the potential use of xantholysin A and C as antimicrobial compounds and its synergistic effect in combination with other antibiotics.

III. RESULTS

Chapter 1

Identification of new residues involved in intramolecular signal transmission in a prokaryotic transcriptional repressor

Molina-Santiago, C., Daddaoua, A., Fillet, S., Krell, T., Morel, B., Duque, E., and Ramos, J.L. (2014) Identification of new residues involved in intramolecular signal transmission in a prokaryotic transcriptional repressor. *J Bacteriol* **196**: 588-594.

Summary

TtgV is a member of the IclR family of transcriptional regulators. This regulator controls its own expression and that of the *ttgGHI* operon, which encodes an RND efflux pump. TtgV has two domains: a GAF-like domain harboring the effector-binding pocket and a helix-turn-helix (HTH) DNA-binding domain, which are linked by a long extended helix. When TtgV is bound to DNA, a kink at residue 86 in the extended helix gives rise to 2 helices. TtgV contacts DNA mainly through a canonical recognition helix, but its three-dimensional structure bound to DNA revealed that two residues, R19 and S35, outside the HTH motif, directly contact DNA. Effector binding to TtgV releases it from DNA; when this occurs, the kink at Q86 is lost and residues R19 and S35 are displaced due to the reorganization of the turn involving residues G44 and P46. Mutants of TtgV were generated at positions 19, 35, 44, 46, and 86 by site-directed mutagenesis to further analyze their role. Mutant proteins were purified to homogeneity, and differential scanning calorimetry (DSC) studies revealed that all mutants, except the Q86N mutant, unfold in a single event, suggesting conservation of the three-dimensional organization. All mutant variants bound effectors with an affinity similar to that of the parental protein. R19A, S35A, G44A, Q86N, and Q86E mutants did not bind DNA. The Q86A mutant was able to bind to DNA but was only partially released from its target operator in response to effectors. These results are discussed in the context of intramolecular signal transmission from the effector binding pocket to the DNA binding domain.

Introduction

Efflux pumps of the RND (resistance-nodulation-cell division) family mediate resistance and tolerance to a wide range of toxic molecules in Gram-negative bacteria. *Pseudomonas putida* DOT-T1E exhibits an extremely high tolerance to solvents due to the synergic action of three RND efflux pumps (Ramos *et al.*, 1995; Duque *et al.*, 2001), called TtgABC, TtgDEF, and TtgGHI that expel organic solvents from the cells (Isken and de Bont, 1996; Kieboom *et al.*, 1998; Ramos *et al.*, 1998; Rojas *et al.*, 2001; Ramos *et al.*, 2002; Fernández *et al.*, 2012). The TtgGHI efflux pump is, from a quantitative point of view, the main toluene extrusion element in DOT-T1E (Rojas *et al.*, 2001; Rojas *et al.*, 2003), and the control of the expression of this efflux pump is mediated by the *ttgV* gene product.

The *ttgV* gene and the *ttgGHI* operon are transcribed divergently, and their promoters overlap, so that binding of TtgV to its operator represses the transcription from its own promoter and that of the *ttgG* promoter (Guazzaroni *et al.*, 2004). TtgV, a member of the IclR family of regulators (Molina-Henares *et al.*, 2006), binds a number of aromatic compounds known as effectors, including 1-naphthol and indole (Guazzaroni *et al.*, 2004; Guazzaroni *et al.*, 2005).

TtgV was previously purified to homogeneity, and a number of biochemical assays revealed that it is a tetramer (Guazzaroni *et al.*, 2005). The three-dimensional structures of TtgV in its apoprotein form and when bound to its target DNA operator were solved by Lu *et al.* (2010). The co-crystal protein-DNA complex revealed that the numerous interactions between TtgV and DNA induced significant distortions within the DNA major groove, which lead to a curvature of 60° at the promoter region (Figure 1A) (Lu *et al.*, 2010). A number of direct interactions between DNA and TtgV take place through amino acid residues R47, T49, R52, and L57, which form part of a helix-turn-helix motif (HTH) (Fillet *et al.*, 2009; Lu *et al.*, 2010). The TtgV/operator co-crystal structure also revealed that amino acids S35 and R19, both located outside the HTH motif (Figure 1) (Fillet *et al.*, 2009; Lu *et al.*, 2010), established direct contacts with DNA, although their role in DNA binding was not defined.

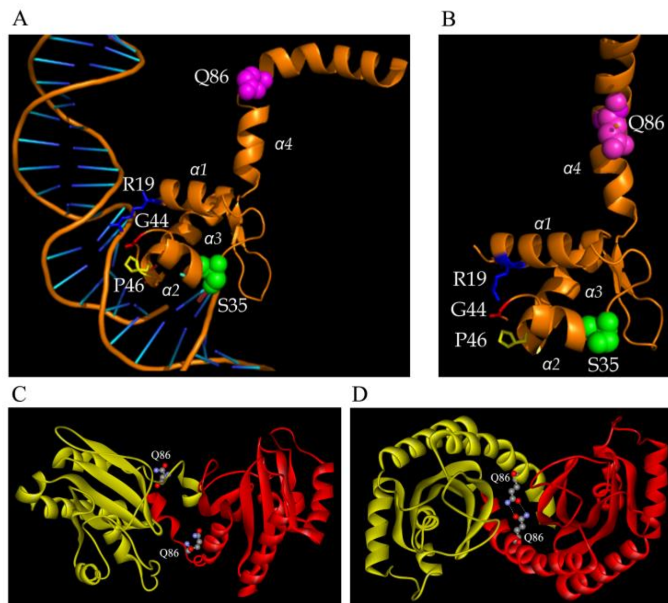

Figure 1. Structural changes induced by the binding of TtgV to DNA. Structure of the TtgV DNA binding domain and the flanking helix α4 in the DNA-bound state (A) and ligand-free state (B). View down the 2-fold axis of DNA-bound (C) and ligand-free (D) TtgV. Glutamine 86 is highlighted. In the DNA-free state, the residues from each monomer of the dimer establish two hydrogen bonds. For clarity purposes, the DNA-binding domain is not shown. The PDB ID of the ligand-free form of TtgV is 2XRN, and that of the DNA-bound form is 2XRO.

Lu *et al.* (2010) showed that in its apoprotein form the two domains of TtgV are aligned through a straight helix; however, when TtgV is bound to DNA, a 90° angle is introduced (Figure 1), breaking the single helix into two smaller helices. This bend occurs at residue Q86 located within the connecting helix (Lu *et al.*, 2010). Apart from being the residue at which the helix kink is introduced, Q86 plays also a central role in establishing intradimer contacts. As shown in Figure 1D, Q86 side chains from the two monomers of the dimer establish two hydrogen bonds; DNA binding causes these bonds to break. Comparison of the three-dimensional structures of apoproteins TtgV and TtgV bound to DNA revealed that the turn of the HTH motif facilitates the interactions between S35, R19, and DNA and that residues G44 and P46 within this turn region are involved in the process (Figure 1). This study aimed to determine the role of residues 19, 35, 44, 46, and 86 in TtgV regulator function. Thus, mutants in these residues were characterized physicochemically in the presence of 1-naphthol and DNA to gauge their effect on effector and DNA binding.

Materials and methods

Site-directed mutagenesis

TtgV mutants were generated by amplification of the *ttgV* gene in plasmid pET28b:TtgV using Pfu turbo DNA polymerase (Stratagene) and 39-mer overlapping primers that incorporated appropriate mismatches to introduce the desired mutations (Daniels *et al.*, 2010). PCR product corresponding to the full plasmid with the expected mutations was digested with DpnI and transformed into *Escherichia coli* BL21(DE3). The TtgV mutant proteins were produced and purified to homogeneity using the protocol described by Fillet *et al.* (2009).

Isothermal titration calorimetry

Microcalorimetric experiments were carried out at 25 ºC using a Valerian-Plotnikov (VP)-microcalorimeter (Microcal, Amherst, MA). Protein and substrates were dialyzed against 25 mM Tris-acetate (pH 8), 100 mM NaCl, 8 mM magnesium acetate, 1 mM dithiothreitol (DTT), and 10% (vol/vol) glycerol. Typically, a 4.8-μl aliquot of a 500 μM 1-naphthol solution, serving as an effector, was injected into a 40 μM solution of TtgV (1.2 ml) or the corresponding mutants. All data were corrected using the heat changes arising from injection of the effector into buffer. Data were analyzed using the "one binding site model" of the MicroCal version of Origin. Titration curves were fitted by a nonlinear least-squares method to a function for the binding of one molecule of substrate to one molecule of target protein.

The parameters ΔH (enthalpy) and K_A (binding constant, $K_A = 1/K_D$, where K_D is the equilibrium dissociation constant) were determined from the fit of the curve. The changes in free energy (ΔG) and in entropy (ΔS) were calculated from the values of K_A and ΔH, using the equation $\Delta G = -RT \ln K_A = \Delta H - T\Delta S$, where R is the universal molar gas constant and T is the absolute temperature (Krell, 2008).

Differential scanning calorimetry

Differential scanning calorimetry experiments were carried out using a VP differential scanning calorimeter (DSC), a capillary-cell microcalorimeter from MicroCal (Northampton, MA), at a scan rate of 60 ºC/h from 5 ºC to 85 ºC. Protein samples were dialyzed for 48 h in 20 mM PIPES [piperazine-N,N=-bis(2-

ethanesulfonic acid)], 8 mM magnesium acetate, 150 mM KCl, 1 mM Tris (2-carboxyethyl) phosphine (TCEP), pH 7.2. Dialyzed samples were centrifuged for 10 min at 14,000 x g in a cold room. Then, the concentration of the protein in solution was determined at 280 nm, using an extinction coefficient of 7,575 M^{-1} cm^{-1} (using the Protparam tool; Expasy). When indicated, 1-naphthol was added to reach a final concentration of 33 or 250 μM, in the latter case to ensure that full saturation of the regulator was reached in each DSC scan.

Calorimetric cells (operating volume, 0.134 ml) were kept under an excess pressure of 60 lb/in^2 bar to prevent degassing during the scan. Several buffer-buffer baselines were obtained before each run with protein solution in order to ascertain proper equilibration of the instrument. Reheating was not performed, as previous studies on TtgV have proved that the denaturation of the protein leads to white precipitates and the unfolding process is therefore not reversible (Fillet *et al.*, 2011).

Circular dichroism

Protein samples were thawed and centrifuged at 14,000 x g for 10 min. Supernatant protein concentrations were determined by measuring absorbance and adjusted to a concentration of 40 μM. 1-Naphthol was added from a 100-fold-concentrated stock solution to obtain final concentrations of 33 μM or 250 μM. Midpoint temperatures (Tm) values were obtained from the midpoint transition of the denaturation curves at 222 nm, and α-helix contents were calculated using the algorithm described by Luo and Baldwin (1997).

EMSAs

For electrophoretic mobility shift assays (EMSAs), DNA fragments containing the *ttgGHI* promoter were amplified using pGG1 as a template with the primer pair 5'-NNNNNNGAATTCGTTCATATCTTTCCTCTGCG-3' and 5'-NNNNNNCTGCAGGGGGATTACCCGTAATGCAC-3'. Fragments were isolated from agarose gels and end labeled with [-32P]deoxy-ATP using the T4 polynucleotide kinase. A 10 μl sample containing about 2 nM labeled DNA (1.5104 cpm) was incubated with various concentrations of purified TtgV for 15 min in 10 μl of binding buffer (50 mM Tris-HCl [pH 7.5], 10 mM NaCl, 0.5 M magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 5% [vol/vol] glycerol) containing 20 μg ml^{-1} of poly(dI-dC) and 200 μg ml^{-1} bovine serum albumin. The

DNA-protein complexes were resolved by electrophoresis in 4% (wt/vol) non-denaturing polyacrylamide gels in 1 x Tris-borate-EDTA buffer (TBE) as previously described (Rojas *et al.*, 2003; Sasse and Gallagher, 2004).

β-galactosidase assays

Cultures were inoculated with bacterial cells from fresh LB agar plates and grown overnight at 30 ºC on LB medium with appropriate antibiotics. Cultures were diluted to an initial turbidity at 660 nm of 0.05 in the same medium supplemented or not with 1-naphthol (1 mM) dissolved in dimethyl sulfoxide (note that the latter did not interfere with the induction assays in this study). β-galactosidase activity was determined in triplicate for permeabilized cells when cultures reached a turbidity at 660 nm of 0.5 (Miller, 1972). The results reported are the means of nine assays.

Results

Residue Q86 is involved in the structural reorganization of TtgV in response to effectors influencing the stability of the protein

TtgV proteins with mutations in residues 86, 46, 44, 35, and 19 were generated by site-directed mutagenesis with the aim of studying the role of these residues in the repression function of the protein. At all positions, we replaced the corresponding residue by alanine. In addition, at position 86, which is located at the kink in the α-helix contacting both domains, we replaced Q by N and G to test the effect of the size of the lateral side chain residue and replaced Q by E to check the effect of the lateral side residue charge. The four mutant variants at residue 86 and the other four alanine mutants at residues 46, 44, 35, and 19 were purified as homogeneous proteins at concentrations of 10 mg ml^{-1} and were stable at 4 ºC. Circular dichroism (CD) analyses were carried out in the presence and in the absence of different concentrations of 1-naphthol. We found that all mutants, in the presence and in the absence of 33 μM and 250 μM 1-naphthol, yielded similar CD results with α-helix percentages between 22.5 and 23.7% (see Figure S1 in the supplementary material). To determine the stability of TtgV, we carried out DSC assays to establish the thermal unfolding of TtgV and its variants. TtgV unfolds in a single event with a Tm of 46.3 ºC (Figure 2). Similar results were

obtained with all mutants (Figure 2 and Table 1; see Figure S2 in the supplementary material), except for the Q86N mutant, which unfolds in two events, with Tm of 41 ºC and 52 ºC (Figure 2D). We previously demonstrated that effector binding to TtgV leads to stabilization of the protein (Fillet *et al.*, 2011) and an increase in Tm of the wild-type from 46.3 ºC to 55.2 ºC in the presence of 250 µM 1-naphthol (Figure 2A). In the present study, we similarly observed that the addition of 1-naphthol stabilized all mutant proteins (Table 1). With the Q86N mutant, who unfolded in two events, the presence of 1-naphthol enhanced the stability of the unfolding event at the lower temperature (Tm increased from 41.4 ºC to 46.5 ºC). This suggests that in the Q86N mutant the effector binding domain corresponds to the domain that unfolds at the lower temperature.

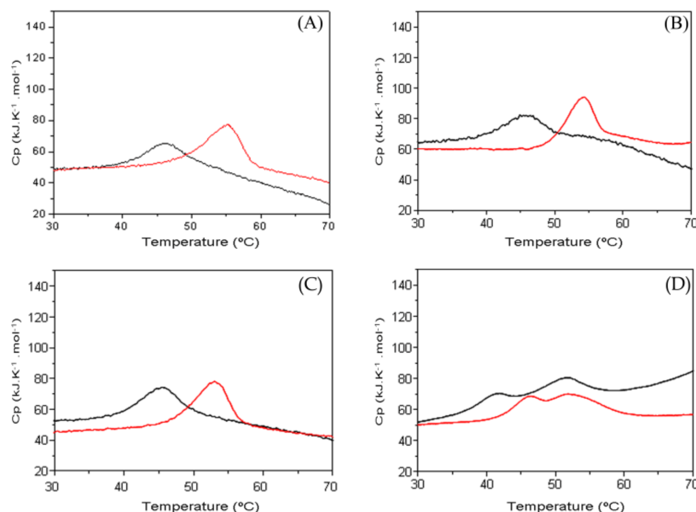

Figure 2. Effects of 1-naphthol on the thermal unfolding of TtgV and mutant variants: wild type (WT) (A) and R19A (B), Q86G (C), and Q86N (D) mutants. For all panels, the DSC experiments of the TtgV mutants were performed in the absence of effector (black line) and in the presence of 250 µM 1-naphthol (red line). Details of the assays are given in Materials and Methods.

Isothermal titration calorimetry (ITC) assays were then carried out to determine if the TtgV mutations affect effector binding. Significant heat changes were observed when 1-naphthol was added to the wild-type protein or any of the mutant proteins (Figure 3). Titration of TtgV mutant proteins (40 µM) with 0.5 mM 1-naphthol produced large exothermic heat changes (Q86A mutant; Figure 3B), confirming that the TtgV mutants recognized 1-naphthol. The wild-type TtgV protein bound 1-naphthol with an affinity of approximately 15 µM. The affinity of most TtgV mutants was in the range of 7 to 20 µM, although it should

be noted that an increase in affinity for 1-naphthol was observed for the G44A (3.5 μM) (Table 2). Fitting of the integrated and dilution-corrected raw data with the "one binding site model" using Origin software (MicroCal) revealed that binding is, in general, driven by favorable enthalpy changes ($\Delta H = - 7.55 \pm 0.38$ kcal·mol^{-1} for G44A to - 32.7 ± 4.7 kcal·mol^{-1} for Q86E) and counterbalanced, in general, by unfavorable entropy changes.

Table 1. Thermodynamic parameters obtained by integration of the peak obtained from the DSC thermal unfolding assays. Transition temperatures (T_m) and unfolding enthalpies (ΔH_m) are presented for each resolvable DSC profile peak in the presence and in the absence of 250 μM naphthol.

	T_m (ºC)	**ΔH_m (kJ·mol^{-1})**
WT	46.30	177
WT + 1-naphthol	55.19	178
R19A	46.43	147
R19A + 1-naphthol	54.33	192
Q86A	45.88	190
Q86A + 1-naphthol	52.87	225
Q86G	45.57	205
Q86G + 1-naphthol	53.04	194
Q86N	41.4	51.05
	51.33	111
Q86N + 1-naphthol	46.46	73.52
	51.83	122
Q86E	45.72	157
Q86E + 1-naphthol	52.68	198
G44A	49.62	110
G44A + 1-naphthol	50.68	140
P46A	47.23	190
P46A + 1-naphthol	55.30	335

To determine whether mutant proteins bind to DNA, we carried out EMSAs, which showed that Q86N and Q86E mutants were unable to bind DNA, whereas the Q86A and Q86G mutants behaved like the wild-type (Figure 4). In contrast to alanine and glycine, asparagine and glutamine side chains are able to form

hydrogen bonds, and it is plausible that the differential capacity of the different Q86 mutants may be related to the establishment of hydrogen bonds with other amino acids. The inspection of the dimer interface of TtgV (Lu *et al.*, 2010) revealed that the Q86 side chains interact, forming two hydrogen bonds. The introduction of a negatively charged Glu side chain instead of the neutral Gln into the center of the dimer interface is likely to create charge repulsion and structural alterations. Mutants at residues R19 and S35 failed to bind DNA, corroborating their role in DNA interactions as reported from the analysis of the TtgV/operator co-crystal structure (Lu *et al.*, 2010). Mutations of G44 and P46 to alanine had a differential impact on the DNA binding capacity. While the P46A mutant was able to bind promoter DNA, the G44A mutant was devoid of DNA binding ability. When EMSAs of the mutant variants that bound DNA were carried out in the presence of 1-naphthol, all of them were released from the DNA (Figure 4). This set of results indicated that in addition to residues in the HTH, which bind the operator, additional residues such as R19 and S35 are critical for DNA binding, mainly because the target operator is bent during binding, allowing alternate contact points.

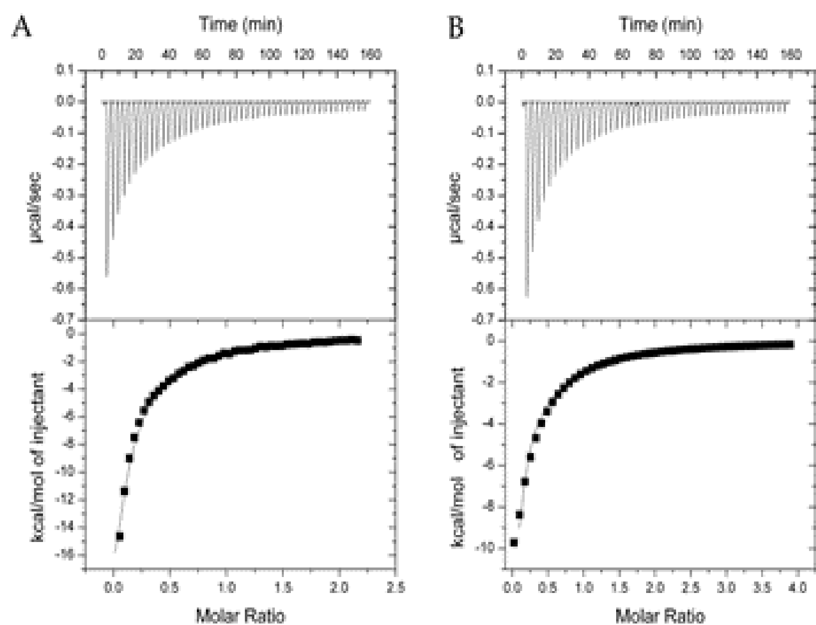

Figure 3. Microcalorimetric titration of TtgV with 1-naphthol. (Upper panels) Raw data for the injection of 4.8 µl aliquots of 0.5 mM 1-naphthol into 40 µM TtgV wild-type (A) and Q86N mutant (B). (Lower panels) Integrated, dilution corrected, and protein concentration normalized peak areas of the raw data. Data were fitted using the “one binding site model” of MicroCal Origin.

TtgV mutant release from target DNA was analyzed using EMSA. TtgV release from DNA involves interaction of the regulator with the effector, as well as intramolecular signal transmission (Figure 5). We found that the wild-type and all mutants are released after effector binding according to a hyperbolic curve with increasing effector concentration. For the P46A mutant, we found that a concentration of 0.87 mM 1-naphthol was required to release 50% of the bound protein, which is a value comparable to that of the wild-type. Interestingly, much higher 1-naphthol concentrations were necessary to trigger the release of the Q86A and Q86G mutants, demonstrating that the Q86 residue is key in the intramolecular signal transmission process.

Table 2. Thermodynamic parameters derived from the microcalorimetric titration of TtgV wildtype and mutants with 0.5 mM 1-naphthol.

Protein	K_d (μM)	K_A (M^{-1})	ΔH ($kcal \cdot mol^{-1}$)	ΔG ($kcal \cdot mol^{-1}$)
WT	15.2±1.9	(6.6±0.5)x10^4	-14.5±0.49	-6.6±0.04
Q86E	10.2±1.3	(9.8±1.2)x10^4	-32.7±4.77	-6.7±0.07
R19A	8.0±0.9	(12.5±1.5)x10^4	-22.4±0.97	-6.8±0.07
Q86G	16.0±0.2	(6.24±4.3)x10^4	-18.2±0.56	-6.5± 0.01
Q86N	6.6±0.5	(15.2±2.0)x10^4	-6.5±0.33	-7.1±0.08
Q86A	20.2±1.3	(4.9±0.3)x10^4	-11.7±0.36	-6.3±0.04
S35A	11.9±0.5	(8.4±0.4)x10^4	-24.4±0.43	-6.6±0.02
P46A	8.2±0.7	(12.2±1.1)x10^4	-27.5±0.85	-6.8±0.04
G44A	3.5±0.6	(28.7±5.3)x10^4	-7.55±0.38	-7.3±0.1

In vivo behavior of TtgV mutants

To test whether the TtgV mutants behaved *in vivo* as deduced from *in vitro* results, we used a fusion of P_{ttgG} to *lacZ* and introduced the TtgV wild-type gene or its mutant variants in *trans* in pBBR (Fillet *et al.*, 2009). Expression from P_{ttgG} was repressed *in vivo* by TtgV, and basal expression increased about 3-fold in response to 1-naphthol, in agreement with previous results (Table 3) (Guazzaroni *et al.*, 2004). Q86A and Q86G mutants behaved like the wild-type, as expected from the *in vitro* assays. Q86E, G44A, R19A, and S35A mutants did not bind DNA, and

consequently expression from P_{ttgG} was high regardless of the presence of 1-naphthol, in consonance with the *in vitro* results (Table 3).

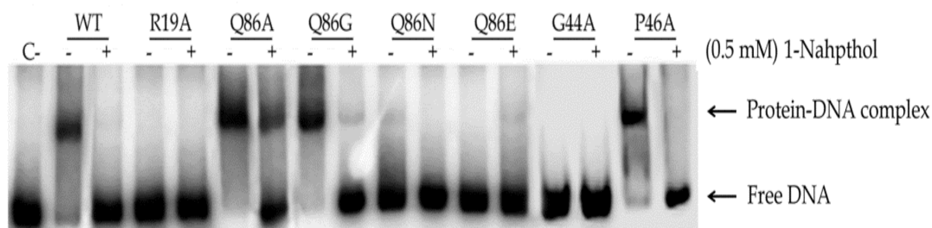

Figure 4. Interaction of TtgV and its mutant derivatives with *ttgGHI* promoter studied by EMSA. A DNA fragment (2 nM) comprising the 295-bp intergenic *ttgV-ttgG* region was incubated without (C-) and with wild-type or mutant TtgV in the absence (-) or in the presence (+) of 1-naphthol. The TtgV proteins were added to reach a concentration of 1 μM, and 1-naphthol was added to a final concentration of 2 mM.

Discussion

The structure of the ligand-free TtgV regulator is composed of two domains that are connected by a long extended helix. Effector binding to the DNA-bound repressor causes major structural changes in which a 90° kink is introduced into this helix, altering the respective positions of the DNA- and effector-binding domains. These structural alterations trigger the release of the DNA-bound protein. Interestingly, the TtgV linker can be classified as glutamine-rich linker or Q-linker, initially described by Wootton and Drummond (1989) as interdomain helices rich in glutamine and glutamate residues. There are a number of studies available that show that Q-linkers are important for efficient interdomain communication (Mattison *et al.*, 2002; Walthers *et al.*, 2003). However, the functional basis for the existence and conservation of Q-linkers is poorly understood.

Here, we show that mutation of Q86 of the TtgV Q-linker has multiple consequences on protein function. This is illustrated by the mutant in which Q86 was replaced by asparagine. Although this amino acid replacement is highly conservative, DSC data show that the mutant unfolds in two separate events instead of a single event for the native protein (Table 1). The same mutant had an increased affinity for 1-naphthol of approximately 3-fold (Table 2), and EMSA

data (Figure 5) showed that the replacement of Q86 renders the protein less efficient in its capacity to mediate its dissociation in response to 1-naphthol.

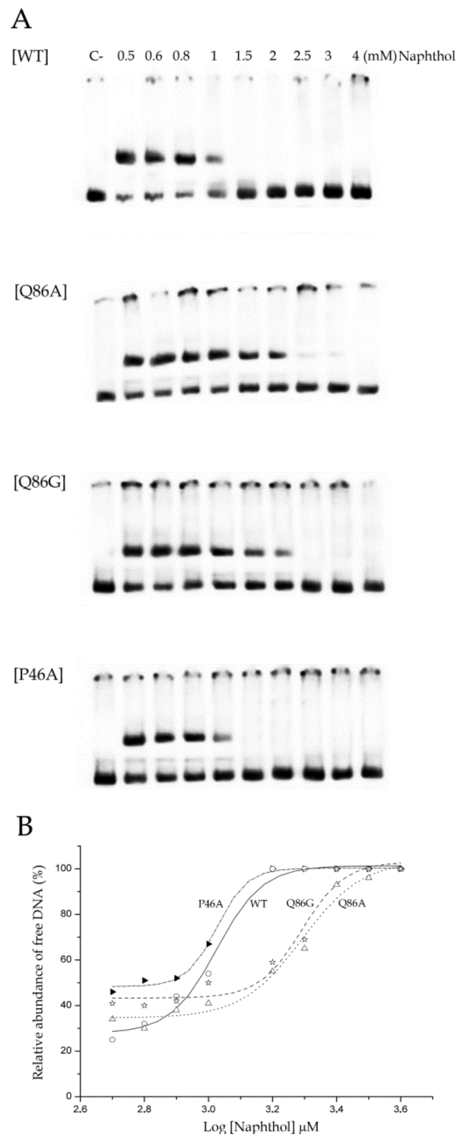

Figure 5. Release of wild-type TtgV and mutants bound to the intergenic *ttgV-ttgG* region in the presence of increasing concentrations of 1-naphthol. EMSAs were carried out as described for Figure 4, except that different concentrations of 1-naphthol were used, and densitometric analyses were performed (using Quantity One software) to determine the amount of DNA released in the presence of increasing concentrations of 1-naphthol with respect to the total shifted DNA in the absence of ligand. (A) EMSAs were carried out with 2 nM of the indicated *ttgV-ttgG* intergenic region (295-bp fragment) (lane C) and 1 µM wild-type or mutant TtgV proteins incubated with increasing concentrations of 1-naphthol (0.5, 0.6, 0.8, 1, 1.5, 2, 2.5, 3, and 4 mM). (B) Representation of the relative abundance of free DNA versus the concentration (log) of 1-naphthol. MicroCal Origin software was used to calculate the relative abundance of free DNA (%) and the EC_{50} of each protein, defined as the ligand concentration for which 50% of the shifted DNA is released.

A plausible structural reason for the central role of Q86 may be its involvement in establishing two intradimer hydrogen bonds (Figure 1). DNA binding would break these bonds and trigger the introduction of a kink in the Q-helix with its apex on Q86. For the four Q86 mutants analyzed, none of the substituent amino acids is likely to form intradimer hydrogen bonds (the two

asparagine side chains are too far removed for mutual interaction), and it can be suggested that the pronounced effects observed for these mutants may be caused by the absence of hydrogen bonds formed between the Q86 substituents.

Parallel to that exist the NarL response regulator, which was also shown to possess a Q-linker. It was shown that NarL phosphorylation causes the separation of both domains caused by a hinge bending of the Q-linker allowing for the otherwise hindered DNA binding (Zhang *et al.*, 2003). Combined data suggest that a functional feature characteristic of Q-linkers may be their capacity to allow kink formation and consequently domain separation.

Q86 is not the only key residue in the TtgV Q-linker. In previous work, we have shown that the formation of an intramonomer salt bridge between R98 and E102 was essential to interdomain communication (Fillet *et al.*, 2011). This salt bridge is formed only in the DNA bound state and is dissolved in the ligand-free form of TtgV (Lu *et al.*, 2010). In analogy to the Q86 mutant, the mutation of both R98 and E102 caused the two domains to unfold in a sequential manner (Fillet *et al.*, 2011).

Two other groups of residues were analyzed in this work: G44 and P46, which form part of the HTH turn, and R19 and S35, which are located outside the HTH motif. The TtgV structure shows that the loop harboring G44 and P46 is the part of the structure farthest removed from the effector binding site. However, mutation of both residues significantly influenced 1-naphthol affinity (Table 2) and also increased the thermal stability of the protein by approximately 1 ºC (P46A) and 3 ºC (G44A). However, the replacement of both residues by alanine had different effects on their DNA-binding capacity. Whereas the replacement of P46 by A had little effect on the protein, the G44A mutant was unable to bind to DNA. Both residues are located on a very sharp turn connecting both helices of the HTH motif, and an amino acid with elevated conformational freedom such as glycine may be required at this position to guarantee the formation of this turn.

The main determinants for DNA binding are usually located within the HTH motif. The TtgV structure shows that R19 and S35 interact directly with neighboring DNA phosphate moieties. Here, we show that mutation of two residues that are located earlier in the protein sequence than the HTH motif abolished DNA binding. This feature may be due to the fact that TtgV binding causes strong DNA distortion, enabling other contacts with DNA.

Table 3. Regulated expression from the P_{ttgG} promoter mediated by TtgV or its mutant variants. Assays were carried out as described in Materials and Methods. Activity is expressed in Miller Units.

Regulator	Without 1-naphthol	+ 1-naphthol
None	3755 ± 280	3980 ± 250
TtgV	1325 ± 120	3900 ± 220
TtgVQ86A	2115 ± 120	3500 ± 150
TtgVQ86G	1355 ± 125	3660 ±200
TtgVQ86E	3830 ± 260	4120 ± 100
TtgVS35A	3890 ± 300	4500 ± 170
TtgVR19A	4240 ± 250	4225 ± 205
TtgVG44A	4250 ± 160	4230 ± 225

TtgV belongs to the IclR family of transcriptional regulators (Molina-Henares *et al.*, 2006), and we have aligned the 50 most similar members of the IclR family to TtgV to determine whether the residues at positions 19, 35, 44, 46, and 86 were conserved and if they played a general role in this family or, if not conserved, whether they played a more specific role in TtgV. The alignment revealed (see Figure S3 in the supplementary material) that the residues equivalent to S35 (50 of 50), R19 (49 of 50), and P46 (46 of 50) were much conserved, whereas at position G44 and Q86 there was a low degree of conservation. This suggests that S35 and R19 could also play a role in DNA binding in other regulators of this family. Our results also reveal that residues 44 and 86 likely play specific roles in TtgV as a repressor.

In summary, we identify here a key glutamine residue in a Q-linker of an IclR-family transcriptional regulator and provide evidence that amino acids outside the HTH are essential for DNA binding.

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Supplementary material

Figure S1. CD analysis of: A) TtgV wild-type; B) Q86A; C) Q86G; D) Q86E; E) Q86N; F) S35A; G) R19A; H) G44A and I) P46A TtgV mutants derived from CD analysis of the protein variants.

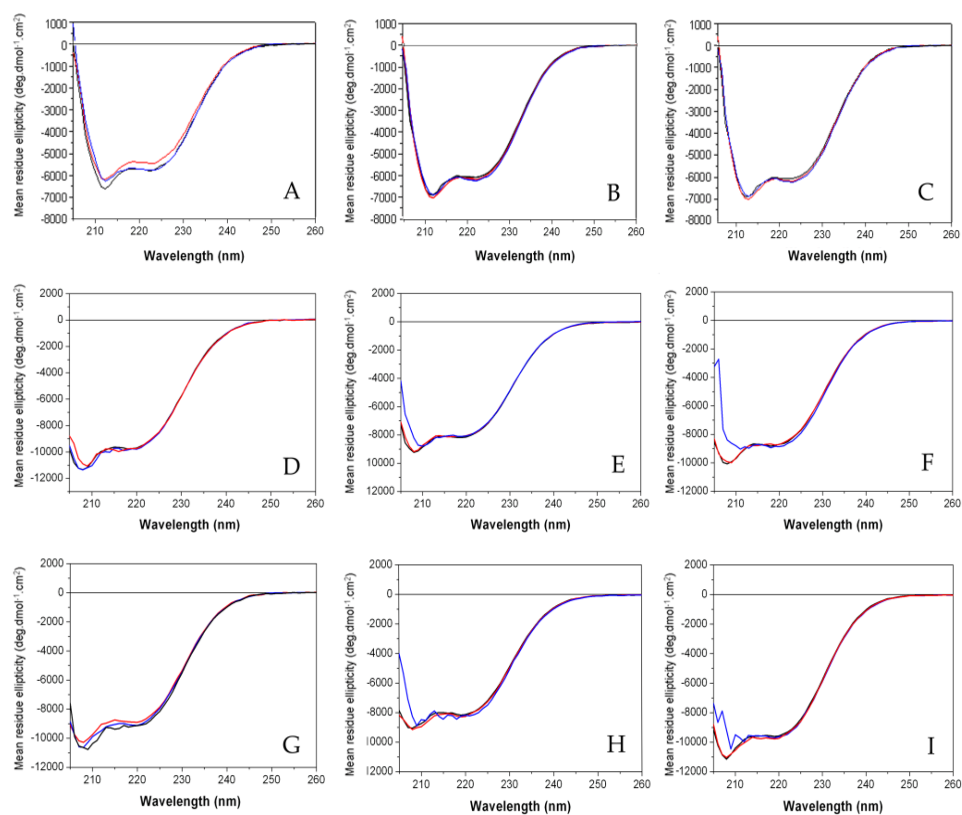

Figure S1. CD analysis of: A) TtgV wild-type; B) Q86A; C) Q86G; D) Q86E; E) Q86N; F) S35A; G) R19A; H) G44A and I) P46A TtgV mutants derived from CD analysis of the protein variants. α-Helix composition was determined at a final concentration of 0.5 mg ml^{-1} in the absence (black) or in the presence of 33 μM (red) and 250 μM 1-naphthol (blue).

Figure S2. Effect of 1-naphthol on the thermal unfolding of TtgV mutants A) Q86A; B) Q86E; C) G44A and D) and P46A.

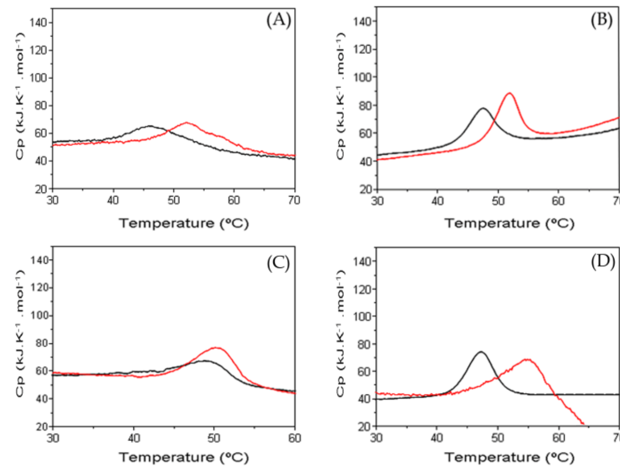

Figure S2. Effect of 1-naphthol on the thermal unfolding of TtgV mutants A) Q86A; B) Q86E; C) G44A and D) and P46A. For all panels, the DSC experiments of the TtgV mutants were performed in the absence (black line) and in the presence of 250 µM (red line) 1-naphthol.

Figure S3. Multialignment of the N-terminus end of TtgV homologues.

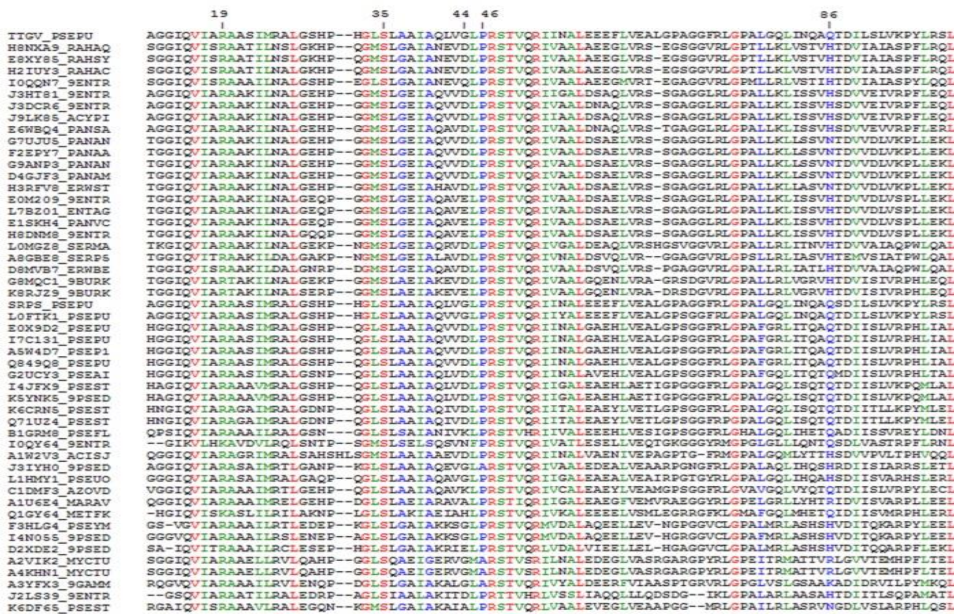

Figure S3. Multialignment of the N-terminus end of TtgV homologues. Sequences were retrieved by BLAST search in Swiss Prot+TREMBL database using the PHL server (htpp://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npa_blast) with default settings. Sequences with a score value lower than E-40 were aligned. The positions corresponding to R19, S35, G44, P46 and Q86 are highlighted.

Chapter 2

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

Molina-Santiago, C., Daddaoua, A., Fillet, S., Duque, E., and Ramos, J.L. (2014) Interspecies signaling: *Pseudomonas putida* efflux pump TtgGHI is activated by indole to increase antibiotic resistance. *Environ Microbiol* **16**: 1267-1281.

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. In *Pseudomonas putida*, the TtgABC efflux pump is the main antibiotic extrusion system that responds to exogenous antibiotics through the modulation of the expression of this operon mediated by TtgR. The plasmid-encoded TtgGHI efflux pump in *P. 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. Because indole is not produced by *Pseudomonas*, the indole-dependent antibiotic resistance seems to be part of an antibiotic resistance program 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 lead to cell death. Co-culture of *E. coli* and *P. putida* Δ*ttgABC* allowed growth of the *P. putida* mutant in the presence of ampicillin because of induction of the indole-dependent efflux pump.

Introduction

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 cell 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 Table S1), 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 does not recognize antibiotics as effectors but indole, a molecule that is not synthesized by

Pseudomonas (Udaondo *et al.*, 2012), and that has been reported to act as an efficient effector for TtgV.

Indole has been proposed to act as an intracellular and intercellular signaling 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 *E. 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 intraspecies 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 *S. enterica* (Nikaido *et al.*, 2008; Vega *et al.*, 2013), favors biofilm formation in *P. aeruginosa*, *P. fluorescens* and *Burkholderia unamae* (March and Bentley, 2004; Lee *et al.*, 2007; Ueda and Wood, 2009; Vert and Chory, 2011; Kim *et al.*, 2013), and attenuation of virulence in *P. 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 76 genes in *P. putida*, which are involved in cell metabolism, cell wall biosynthesis and stress defense. These results support indole's role as a signaling molecule in *P. 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.

Materials and methods

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 LB medium at 30 ºC (*P. putida*) or 37 ºC (*E. coli*) 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^{-1} kanamycin and 20 μg ml^{-1} 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 of 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 ($1 \pm 0.1 \times 10^5$ CFU ml^{-1}) with sterile LB broth, and then 10 ml of *E. coli* cells were mixed with 10 ml of *P. putida* DOT-T1E or *P. putida* DOT-T1E-18. Pure culture experiments for each strain were also conducted as controls. When indicated, ampicillin (300 μg ml^{-1}) was added to all cultures and subsequently incubated for 20 h with agitation at 30 ºC. Samples were removed at the end of the assay and serial dilutions plated on selective media. Solid LB medium supplemented with 20 μg ml^{-1} rifampicin was used to enumerate *P. putida* DOT-T1E; solid LB with rifampicin and kanamycin was used to count *P. putida* DOT-T1E-18, and LB supplemented with 50 μg ml^{-1} of streptomycin and 100 μg ml^{-1} of ampicillin was used to count *E. coli* LK111. After 24 h at 30 ºC, colonies were counted, and CFU ml^{-1} were inferred. Each experiment was repeated at least three times.

Minimum inhibitory concentration assays (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 concentrations of the antibiotics used were: tetracycline (10,000 μg ml^{-1}), chloramphenicol (3,000 μg ml^{-1}), norfloxacin (200 μg ml^{-1}), erythromycin (3,000 μg ml^{-1}) and ampicillin (10,000 μg ml^{-1}). At least three independent experiments were carried out for each determination, and each experiment was run in triplicate. The

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, LB agar plates with or without indole (300 μM) were spread with a suspension of approximately 10^8 CFU ml^{-1} 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, Madrid, Spain) were placed on the surface of plates. After 18–20 h at 30 ºC, the inhibition zone (in mm) 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 earlier when pure indole was used.

DNA microarrays

A *P. putida* array (Progenika, Vizcaya, Spain) was used for transcriptomic studies; the array contains 5539 gene-specific oligonucleotides (50-mer) spotted in duplicate onto γ-amino silane-treated 25 x 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 (Trizol)/BCP (1-Bromo-3-Chloropropane) 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., Madrid, 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 (http://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 HPF (Invitrogen, Madrid, Spain) at a concentration of 5 μM and the following PMT (Photomultiplier tubes) 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 of LB supplemented with 5 μM of HPF. Flasks were incubated in a light-insulated shaker at 30 ºC 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^{-1} 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^{-1}), and 10 μl of resuspended culture. In the case of the *E. coli* supernatant, 20 μl of liquid 10X LB 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 420–580 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).

qRT-PCR

The extraction of RNA was performed as previously described. The primers used for real-time PCR analyses are listed in Table S7. Real-time PCR amplification was carried out on a MyiQ2 system (Bio-Rad, Madrid, Spain) 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 MgCl2, SYBR green I, 20 nM fluorescein, stabilizers (Bio-Rad) (0.4 µM for each primer)] and 2 µl template cDNA (diluted 10- or 1000-fold). Thermal cycling conditions were: 1 cycle at 95 ºC for 10 min and then 40 cycles at 95 ºC for 15 s, 61.1 ºC or 61.7 ºC (for indole and ampicillin experiments respectively) for 30 s, and 72 ºC for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. A final extension cycle (72 ºC for 1 min) was performed. The PCR products size were between 132 and 350 bp. Melting curve analysis was performed by gradually heating the PCR mixture from 55 to 95 ºC at a rate of 0.5 ºC 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 (ΔΔ*CT*) method (Pfaffl, 2001).

Results

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

In *P. 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 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, norfloxacin (bactericidal compounds); and chloramphenicol, erythromycin and tetracycline (bacteriostatic antibiotics).

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)

First, we determined the minimum inhibitory concentrations (MICs) of the four strains for the previous five antibiotics. We found that the MICs 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^{-1} chloramphenicol, 400 μg ml^{-1} erythromycin or 8 μg ml^{-1} tetracycline halted cell growth, although 100% of the cells remained viable throughout the assay (3 h) (see Figure 1A for chloramphenicol and Figure S1). For the TtgABC mutant, 70 μg ml^{-1} 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 two orders of magnitude because of cell lysis. For the parental strain DOT-T1E and the PS28 strain, ampicillin at 625 μg ml^{-1} and norfloxacin at 2 μg ml^{-1} 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 (Fillet *et al.*, 2009), 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 as 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.

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

Antibiotic	**DOT-T1E**		**T1E-18**		**T1E-PS28**		**T1E-PS32**	
(μg ml^{-1})	**-**	**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

Kohanski *et al.* (2007) used the fluorescent dye HPF to monitor *in vivo* fluorescence quenching in response to bactericidal and bacteriostatic compounds. We have carried out similar assays and 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 (Figure S1C and E) failed to promote HPF quenching in *P. putida* and its mutants, a result that matches those for *E. coli* (Kohanski *et al.*, 2007). For the wild-type DOT-T1E with ampicillin (Figure 1C) and in lesser degree with norfloxacin (Figure S1G), 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^{-1} were enough to induce the quenching reaction (Figure 1I). This value is three-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 (Figure 1), a role in ampicillin efflux should be ascribed to TtgGHI because 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 the range of antibiotics tested.

Bactericidal compounds do not kill *P. 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 an universal killing mechanism in *E. coli* for bactericidal antibiotics operating through the generation of hydroxyl radicals, regardless of their specific cell targets, based on the 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 *P. 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 if in response to ampicillin, the cells activate the oxidative stress response program. We carried out transcriptomic assays and analyzed the expression of a number of oxidative stress genes using quantitative real-time polymerase chain reaction (qRT-PCR). To this end, total RNA was extracted from DOT-T1E cells exposed to or not exposed to 300 μg ml^{-1} ampicillin and global expression analyses were performed.

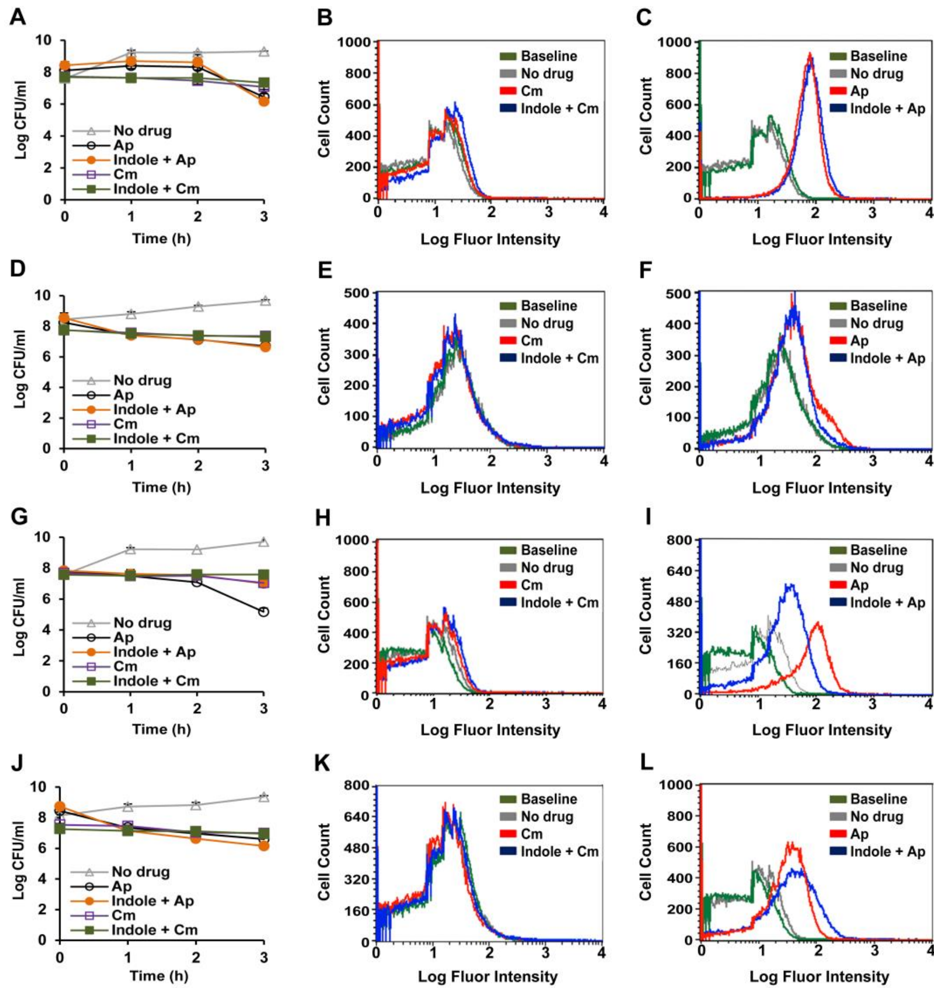

Figure 1. HPF quenching in *P. putida* DOT-T1E and mutants in the presence of indole and antibiotics. (A, D, G, J) Log change in colony-forming units per milliliter (CFU ml^{-1}) by *P. putida* DOT-T1E, T1E-PS28, T1E-18 and T1E-PS32 respectively. Symbols: the no-drug control is represented by grey triangles; 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 ± standard deviation of the mean. (B, E, H, K) HPF quenching 3 h after the addition of the drug chloramphenicol to *P. putida* DOT-T1E (150 μg ml^{-1}), T1E-PS28 (150 μg ml^{-1}), T1E-18 (70 μg ml^{-1}) and T1E-PS32 (2 μg ml^{-1}) cultures. Symbols: the baseline is represented by green line; grey line, control without drug addition; red line, chloramphenicol; and blue line, indole (300 μM) and chloramphenicol. (C, F, I, L) HPF quenching 3 h after the addition of the drug ampicillin to *P. putida* DOT-T1E (300 μg ml^{-1}), T1E-PS28 (300 μg ml^{-1}), T1E-18 (100 μg ml^{-1}) and T1E-PS32 (20 μg ml^{-1}) cultures. Symbols: the baseline is represented by green line; grey line, control without drug addition; red line, ampicillin; and blue line, indole (300 μM) and ampicillin.

We observed that in response to ampicillin, 57 genes increased their expression and 22 genes were repressed (see Table 3, Appendix S1 and Table S2); however, we found that 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), 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 six-fold. In *P. putida* DOT-T1E-18, which exhibited enhanced sensitivity to ampicillin, no induction of oxidative stress genes was observed either in response to ampicillin (Appendix S1 and Tables S3 and S4). 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.

Table 3. *Pseudomonas putida* DOT-T1E genes induced in presence to 300 μg ml^{-1} 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 conversión	
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

Indole is an interspecies signaling molecule

Genome analysis of DOT-T1E did not reveal the presence of tryptophanase orthologues 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 high-pressure liquid cromatography - mass spectrometry (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.

Because indole has been described as an interspecies signaling 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 Luria–Bertani (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 and T1E-PS28 (Figure 2A and C) exhibited a similar level of resistance to antibiotics in the presence and in the absence 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^{-1} of ampicillin inhibited the growth of DOT-T1E-18, the addition of *E. coli* LK111 culture supernatant was sufficient to alleviate this effect, although a short delay was observed before the cells entered log phase and growth rates were 20% slower than in the absence of antibiotics.

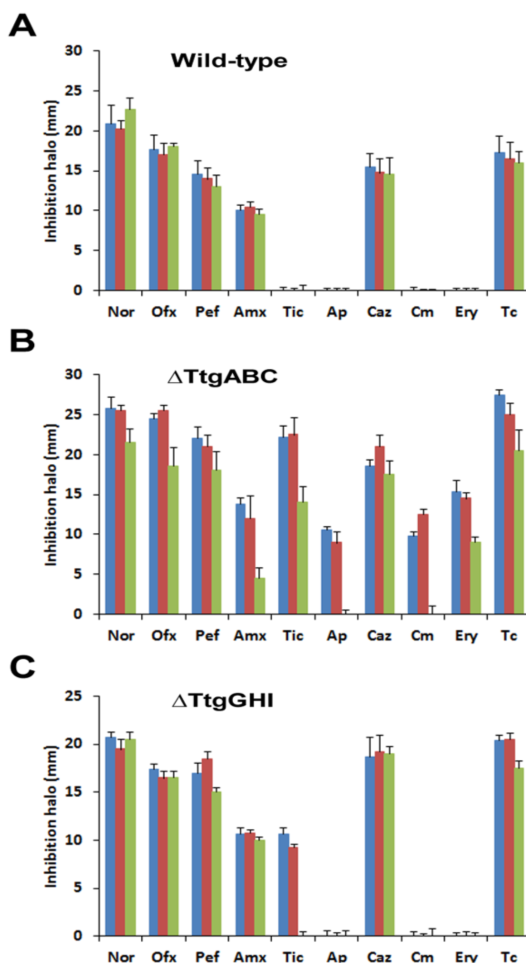

Figure 2. Effect of *E. coli* K12 LK111 supernatant on antibiotic resistance in the *P. putida* DOT-T1E, T1E-18 and T1E-PS-28 strains. Inhibition halos (in mm) produced by several antibiotics. From left to right and top to bottom: norfloxacin, ofloxacin, pefloxacin, amoxicillin, ticarcillin, ampicillin, ceftazidime, chloramphenicol, erythromycin and tetracycline in the absence (blue) and in the presence of cell-free culture supernatant of indole producing *E. coli* cells (green) and in the presence of cell-free culture supernatant of non-indole producing *E. coli* cells (red).

A. *P. putida* DOT-T1E.

B. *P. putida* T1E-18.

C. *P. putida* T1E-PS28.

We subsequently tested the *in vivo* growth of *P. putida* wild-type, DOT-T1E-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^{-1}), and growth was carried out in LB without antibiotics or with ampicillin. As controls, each of the strains was 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^{-1}, 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 earlier co-culture assays but in the presence of 300 μg ml^{-1} ampicillin. *Escherichia 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).

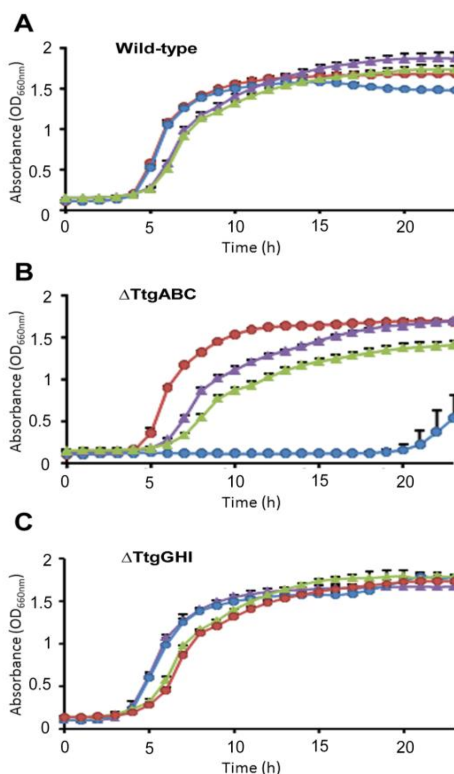

Figure. 3. *Escherichia coli* supernatant effect on the growth of *P. putida* DOT-T1E, T1E-18 and T1E-PS28 strains in the presence and absence of ampicillin. *Pseudomonas putida* DOT-T1E, T1E-18 and T1E-PS28 were grown overnight at 30 ºC in LB liquid medium. The cultures were diluted in LB medium or spent-LB medium in which E. coli LK111 had been grown (this medium contained about 400 μM indole), in the latter case 10 x LB was added to reach a 1 x LB concentration. Half of the samples were used as a control and to the other 100 μg ml^{-1} Ap was added. Growth was followed by measuring the turbidity every hour using a Bioscreen for 24 h.

A. Wild-type strain.

B. *Pseudomonas putida* DOT-T1E-18.

C. *Pseudomonas putida* DOT-T1E-PS28.

Legend: control LB (red circle); LB with ampicillin (blue circle); *E. coli* supernatant (purple triangle); *E. coli* supernatant and ampicillin (green triangle).

Co-cultures of *E. coli* and DOT-T1E cells and of *E. coli* and T1E-18 were prepared with 10^5 CFU ml^{-1} of each strain. We found that T1E cell density after 8 h was in the range of 10^8 CFU ml^{-1} 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 signaling is functional and that *P. putida* proliferates in media with antibiotics because of the presence of a chemical signal produced by *E. coli*.

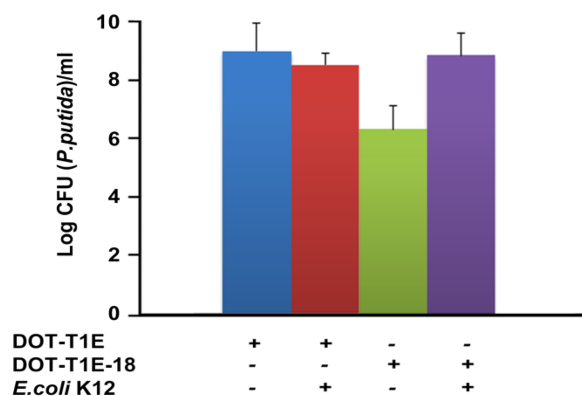

Figure 4. Growth of *P. putida* strains in the presence of *E. coli* K-12 LK111 and ampicillin. *Pseudomonas putida* DOT-T1E and T1E-18 were grown with 300 μg ml^{-1} ampicillin in the presence and in the absence of *E. coli* K-12 LK111 and were selected in LB with Rif and in LB with Km as indicated in the text. The figure represents the CFU of *P. putida* DOT-T1E or T1E-18 per ml of culture in the presence and in the absence of *E. coli* K-12.

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

Because the earlier series of results supported that indole is a potential secondary signal molecule in intraspecies and interspecies 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 Table S5) 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 not 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 that may be related to the need to build cytochromes for the Nuo respiratory chain. Some enzymes related to oxygen stress such as 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 three-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 five 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.

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

Equivalent assays to those described earlier 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 *P. putida*

In *P. 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 compounds extrusion is clear because the T1E-18 mutant exhibited increased antibiotic sensitivity to the tested antibiotics compared with 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 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 *P. 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 auto-fluorescence, which was indeed not the case with bacteriostatic antibiotics such as chloramphenicol and tetracycline (see Figure 1). That quenching of HPF, related to the presence of bactericidal compounds, is clear because in the T1E-18 mutant, devoided 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 devoided of both TtgABC 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.

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

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

In enterobacteria, indole behaves as an intraspecies signaling molecule because it increases resistance to bactericidal (ampicillin and norfloxacin) and bacteriostatic (chloramphenicol, tetracycline and erythromycin) antibiotics. Indole is also an

interspecies signaling 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 an interkingdom signal.

A characteristic of a molecule to be considered as a signaling 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 signaling 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 76 genes in the wild-type *P. putida* DOT-T1E (43 induced and 23 repressed, Table 4 and Table S5). Although indole at high concentrations can delay growth of *P. putida*, this microorganism can grow exponentially in the presence of up to 1.5 mM indole in the culture medium. 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 eight 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 signaling 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 signaling 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 *P. 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 signaling, particularly interspecies signaling, can be considered part of the program 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 come the problem of increasing microbial resistance, which results in a dramatic drop in the effectiveness of the therapies. The identification of signaling 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.

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Supplementary material

Appendix S1

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

We explored the sets of genes up-regulated and down-regulated by 300 μg ml^{-1} ampicillin using gene expression microarrays (Tables S2, S3 and S4). 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 two 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 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 tryptophan). Twenty genes, including some from the flagellar system and dipeptidases, were repressed more than 2-fold in response to ampicillin (Table S2).

Since DOT-T1E-18 (Δ*ttgABC*) showed a marked sensitivity to ampicillin, we tested the effect of a sub-lethal concentration of this β-lactam (100 μg ml^{-1} for 3 h) on the global transcriptional pattern of DOT-T1E-18 (Table S3) (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-T1E-18 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, and 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. (Tables S3 and S4).

Figure S1. HPF quenching in *P. putida* DOT-T1E and T1E-18 by indole and antibiotics.

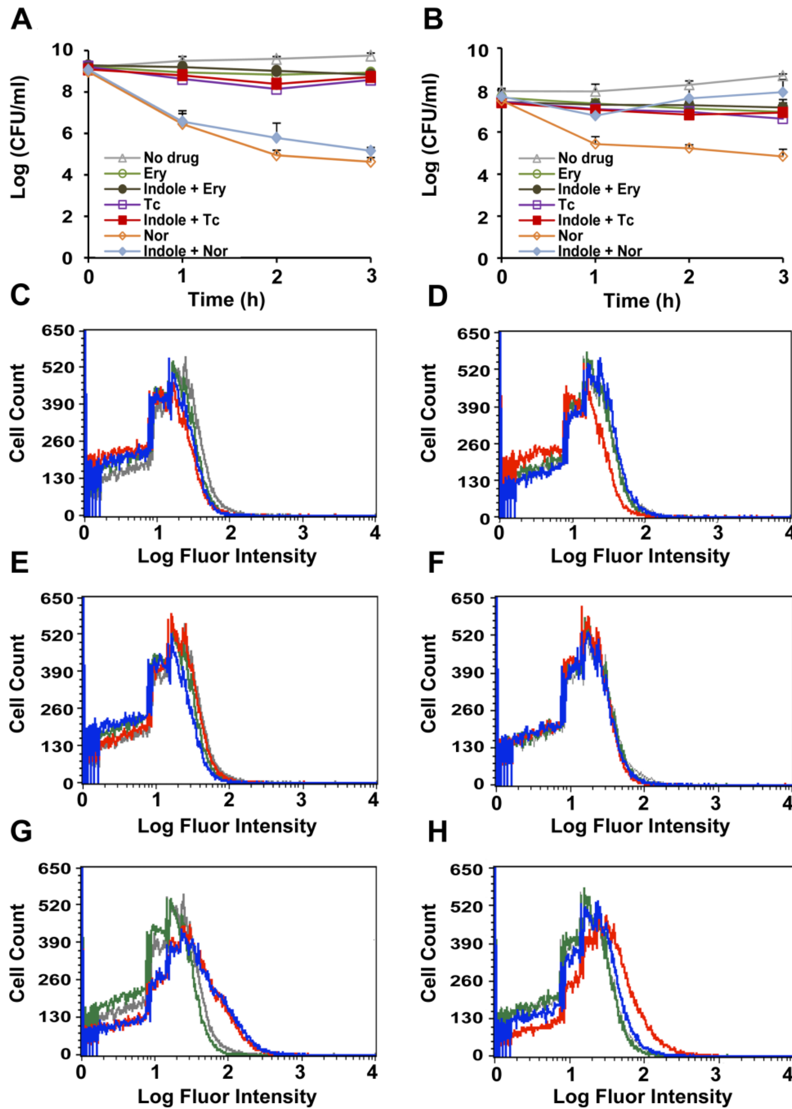

Figure S1. HPF quenching in *P. putida* DOT-T1E and T1E-18 by indole and antibiotics. (A, and B) Log change in colony-forming units per milliliter (cfu/ml). No-drug control is represented by grey triangles. (C, D, E, F, G and H) HPF quenching. Representative measurements are shown and were taken 3 h 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^{-1} tetracycline [Tc], (E) (200 μg ml^{-1} erythromycin [Ery]) and bactericidal antibiotic (G) (1 μg ml^{-1} 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^{-1} tetracycline [Tc], (F) (100 μg ml^{-1} erythromycin [Ery]) and bactericidal antibiotic (H) (0.5 μg ml^{-1} norfloxacin [Nor]) in *P. putida* DOT-T1E respectively.

Table S1. *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	CzcB2	T1E_5271

	subunit		
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	

Table S2. *Pseudomonas putida* DOT-T1E genes repressed in presence of 300 μg ml^{-1} 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

Table S3. *Pseudomonas putida* T1E-18 genes induced in presence of 100 μg ml^{-1} 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 oxigenase	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

Table S4. *Pseudomonas putida* T1E-18 repressed genes in presence of 100 μg ml^{-1} 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

Table S5. *Pseudomonas 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

Table S6. *Pseudomonas 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

Table S7. 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

Chapter 3

Differential transcriptional response to antibiotics by *Pseudomonas putida* DOT-T1E

Molina-Santiago, C., Daddaoua, A.; Gómez-Lozano, M., Udaondo, Z., Molin, S., and Ramos, J.L. (2014) Differential transcriptional response to antibiotics by *Pseudomonas putida* DOT-T1E. Under review at Nucleic Acid Research.

Summary

Multi-drug resistant bacteria are a major threat to humanity, especially because the current battery of known antibiotics is not sufficient to combat infections produced by these microbes. Therefore, the study of how current antibiotics act and how bacteria defend themselves against antibiotics is of critical importance. *Pseudomonas putida* DOT-T1E exhibits an impressive array of RND efflux pumps, which confer this microorganism an unusually high resistance to organic solvents and antibiotics that would kill most other microorganisms. We have chosen DOT-T1E as a model microbe to study the microbial responses to a wide battery of antibiotics (chloramphenicol, rifampicin, tetracycline, ciprofloxacin, ampicillin, kanamycin, spectinomycin and gentamicin). RNA-seq analyses revealed that each antibiotic provokes a unique transcriptional response profile in DOT-T1E. While many of the genes identified were related to known antibiotic targets, others were unrelated or encoded hypothetical proteins. These results indicate that our knowledge of antibiotic resistance mechanisms is still partial. We also identified 138 new sRNAs in DOT-T1E, dramatically adding to the 16 that have been previously described. Importantly, our results reveal that a correlation exists between the expression of mRNA and sRNA, indicating that some of these sRNAs are likely involved in fine tuning the expression of antibiotic resistance genes. Taken together, these findings open new frontiers in the fight against multi-drug resistant bacteria and point to the potential use of sRNAs as novel antimicrobial targets.

Introduction

Pseudomonas putida strains are ubiquitous microorganisms that exhibit a broad metabolic versatility, which allows them to adapt and survive under different nutritional status in edaphic and aquatic environments (Dos Santos *et al.,* 2004; Moore *et al.,* 2006). We have focused on strain DOT-T1E, an isolate of a wastewater treatment plant that has the ability to thrive in the presence of high concentrations of organic solvents such as aromatic hydrocarbons (Ramos *et al.,* 1995). It was also found that this strain is resistant to a number of toxic compounds (dyes, ionophores) and a broad range of antimicrobial compounds (Ramos *et al.,* 1998; Terán *et al.,* 2003; Fernández *et al.,* 2012; Fillet *et al.,* 2012). Antibiotic resistance is a major concern in the field of medicine, not only because opportunistic pathogens are developing resistance against clinically important antibiotics (D'Costa *et al.,* 2006; Arias and Murray, 2009), but also because antibiotic resistance occurs in a number of saprophytic microbes, such as *P. putida*, that may cause nosocomial infections in immunocompromised patients (Molina *et al.,* 2014).

The main mechanism of resistance to drugs and antibiotics in bacteria of the genus *Pseudomonas* is the extrusion of these chemicals from the cells to the external medium through the action of multidrug efflux pumps belonging to the resistance-nodulation cell division (RND) family (Rojas *et al.,* 2001; Fernández *et al.,* 2012; Molina-Santiago *et al.,* 2014a). Among all of the RND efflux pumps present in DOT-T1E, TtgABC has been shown to be the most relevant in antibiotic extrusion, while a second efflux pump named TtgGHI plays a secondary role (Molina-Santiago *et al.,* 2014a). However, the global response of *P. putida* to antibiotics has been overlooked and only a few genome-wide transcriptional studies based on DNA microarray have been performed (Fernández *et al.,* 2012; Molina-Santiago *et al.,* 2014a). A limitation of previous studies is that the low sensitivity of microarrays only revealed a fraction of the expressed genes (Stjepandic *et al.,* 2002). New genome-wide transcriptional studies are based on high-throughput RNA sequencing (RNA-seq), which allows a more sensitive identification of genes that are transcriptionally active and associated with a particular physiological state (McClure *et al.,* 2013). In addition, this approach permits the identification of small RNAs (sRNAs) and riboswitches that are known to play key roles in gene regulation under stress conditions.

The significance of sRNAs in the context of bacterial regulation is becoming increasingly apparent, and their role in the regulation of a wide range of physiological responses, such as those to pH, solvent stress or temperature shifts are evident since they modulate transcription, translation, mRNA stability, DNA maintenance, or gene silencing. Besides, recent studies have suggested an important role of sRNAs in the bacterial response to antimicrobials (Yu and Schneiders, 2012; Howden *et al.,* 2013).

In this study we analyzed the global gene expression of *P. putida* DOT-T1E growing in the presence of clinically relevant antibiotics belonging to different families, which are directed to different targets, for example cell wall biosynthesis (ampicillin), DNA synthesis (ciprofloxacin), ribosome functions (tetracycline, kanamycin, gentamicin, spectinomycin, and chloramphenicol), and transcriptional machinery (rifampicin) (Fernandes, 2006; Lange *et al.,* 2007). A comparative analysis of RNA-seq data revealed expression of specific genes related to the mechanism of action of the antimicrobial compounds and a number of other genes whose expression varies significantly in response to the tested antibiotic. RNA-seq analysis also revealed 138 novel sRNAs in *P. putida* DOT-T1E that show a differential expression profile in response to antibiotics.

Materials and Methods

Growth conditions and RNA isolation

Single colonies of *P. putida* strain DOT-T1E were grown overnight in Luria–Bertani (LB) medium at 30 ºC. Overnight cultures were then diluted to a starting OD_{600} of 0.01 in the same medium and 50 ml aliquots were dispersed into separate 250 ml Erlenmeyer flasks and incubated with shaking at 200 x rpm. When cultures reached exponential phase (≈0.5 at OD_{600}), antibiotics were added at sub-lethal concentrations to the culture medium to reach a final concentration of 1 μg ml^{-1} kanamycin (Km), 300 μg ml^{-1} ampicillin (Ap), 150 μg ml^{-1} chloramphenicol (Cm), 4 μg ml^{-1} tetracycline (Tc), 0.5 μg ml^{-1} ciprofloxacin (Cip), 300 μg ml^{-1} spectinomycin (Sp), 500 μg ml^{-1} rifampicin (Rif) and 2 μg ml^{-1} gentamicin (Gm). Cultures were then incubated under the same conditions for an additional hour. RNA isolation was done as previously described (Gómez-Lozano *et al.*, 2014). Harvested cells were mixed immediately with 0.2 volumes of STOP

solution (95% [v/v] ethanol, 5% [v/v] phenol) and pelleted by centrifugation. Subsequently, total RNA was extracted with Trizol (Invitrogen). Removal of DNA was carried out by treatment with DNase I (Fermentas) in combination with the RNase inhibitor RiboLock (Fermentas). The integrity and quality of total RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Removal of 23S, 16S and 5S rRNAs

The 23S, 16S and 5S rRNAs were removed by subtractive hybridization using the MICROBExpress kit (Ambion) with modifications (Gómez-Lozano *et al.*, 2014). Capture oligonucleotides complementary to the rRNAs were designed specifically for *P. putida* DOT-T1E (Table S1). Compared with the standard protocol, 25% more capture oligonucleotides and magnetic beads were used. Removal of the rRNA was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Library preparation and RNA sequencing

The sequencing libraries were prepared using the TruSeq Stranded mRNA Sample Preparation kit (Illumina). Each library was prepared with RNA isolated from cells grown in duplicate for each condition. After each step the samples were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies), and the final concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen). The libraries were sequenced using the Illumina HiSeq2000 platform with a paired-end protocol and read lengths of 100 nt.

Data analysis

Computational analysis of RNA-seq data were done using Rockhopper 1.3.0 (McClure *et al.*, 2013), including read mapping, normalization, quantification of transcript abundance and sRNA identificationThe reads were mapped onto the annotated *P. putida* DOT-T1E reference genome (GenBank accession no. NC_018220). An analysis of variance (ANOVA) was performed on the average expression of the mRNAs to determine those with differential expression between two conditions tested (P-value < 0.02 and two-fold change). The Benjamini–Hochberg multiple testing correction was applied (Benjamini *et al.*, 2001) when more than two samples were compared (P-value < 0.05). Heatmaps and hierarchical cluster analysis were created based on expression levels (P-value <

0.05). To be included in the heat map, genes were required to have at least 10 alignments over all samples and the fold change had to exceed 2 or to be less than -2.

Pseudomonas putida DOT-T1E sRNAs were searched against the Rfam database to look for homologies to non-coding RNA (ncRNA) entries. Putative homologues of each novel transcriptional unit were predicted via BLASTN comparison of each sequence to all sequenced bacterial genomes ($E = 10^{-6}$, word = 11). Only hits with nucleotide identity higher than 60% combined with coverage between query and subject sequence higher than 80% were considered to be conserved.

Pseudomonas putida core-genome analysis

Sequences belonging to 154 sRNA were mapped using the Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1990) against the database of the core genome of nine *P. putida* strains (Udaondo *et al.*, unpublished) (*P. putida* BIRD1, *P. putida* DOT-T1E, *P. putida* F1, *P. putida* GB1, *P. putida* Idaho, *P. putida* KT2440, *P. putida* PC9, *P. putida* S16 and *P. putida* W619). BlastN results were parsed using a similarity sequence of 79% and e-value > 0,001.

Gene ontology analysis

Functional annotation of expressed genes was performed using Gene Ontology (GO) (Ashburner *et al.*, 2000) terms using BLAST (Altschul *et al.*, 1990) against UniProt database (UniProt-GO) (Suzek *et al.*, 2007). The GO terms were exported to WEGO GO plotting tool (Ye *et al.*, 2006) and categorized using level 3 of the GO lineage.

RNA-sequencing data accession number

The sequence reads have been deposited in the GEO database under accession no. GSE56749.

Results and discussion

The transcriptional profile of DOT-T1E using RNA-seq

We analyzed the transcriptome of *P. putida* DOT-T1E growing in the presence and absence of different antibiotics using Illumina RNA-seq. We first determined the minimum inhibitory concentrations (MICs) of DOT-T1E using a number of antibiotics belonging to different families that target different cell processes. The MICs were established to be: β-lactam antibiotics (ampicillin, 600 μg ml^{-1}), fluoroquinolone antibiotics (ciprofloxacin, 1 μg ml^{-1}), aminoglycoside antibiotics (kanamycin, 2 μg ml^{-1}; gentamicin, 4 μg ml^{-1}), aminoclytol antibiotics (spectinomycin, 600 μg ml^{-1}), rifamycin antibiotics (rifampicin, >1000 μg ml^{-1}), tetracycline antibiotics (tetracycline, 8 μg ml^{-1}) and phenolic antibiotics (chloramphenicol, 300 μg ml^{-1}). To study the effect of these compounds on the transcriptome of DOT-T1E, we used a concentration that was half of the MIC values. The reference genome sequence for accurate read mapping of the RNA-seq data was the sequence belonging to *P. putida* DOT-T1E, which is deposited in GenBank database (NC_018220). Read mapping against the DOT-T1E chromosome was performed using Rockhopper, which allowed us to identify operons, differentially expressed genes, UTRs and sRNAs. We visually inspected these elements along the chromosome using IGV 2.3. The analysis identified the expression of the 5756 mRNAs which compose the entire transcriptome of DOT-T1E, 58 tRNAs and 154 *trans*-encoded sRNAs with an average size of 174 bp, of which 138 were detected for the first time in DOT-T1E.

Different antibiotics lead to different patterns of mRNA expression

Global mRNA expression profiles for the data sets were analyzed using heatmaps that included genes with a two-fold change with respect to the control condition established in LB medium without antibiotic supplementation (Figure 1).

The analysis of the heatmap images of 604 genes that were differentially expressed revealed specific patterns of response to different antibiotics. Comparison of the transcription patterns of DOT-T1E in the presence of ampicillin, chloramphenicol and kanamycin showed profiles with the lowest variance with respect to those of the control sample without antibiotic; while clear differential expression patterns were seen for the rest of antibiotics based on

variance analyses. The presence of chloramphenicol and kanamycin also led to the up-regulation of a set of genes that were unique and characteristic of each of these antibiotics and were not induced in any of the other conditions tested. Ciprofloxacin showed a unique pattern with the largest group of genes up-regulated and a small group of genes down-regulated compared with the rest of patterns obtained (see below). Gentamicin and rifampicin showed similar patterns between them with the exception of a clear group of up-regulated genes in rifampicin that were not induced by gentamicin. In both conditions, patterns were different to the control condition and to the rest of the antibiotics tested. Finally, spectinomycin and tetracycline showed two characteristic patterns with a very small group of genes up-regulated, with almost no coincidences with the other patterns obtained.

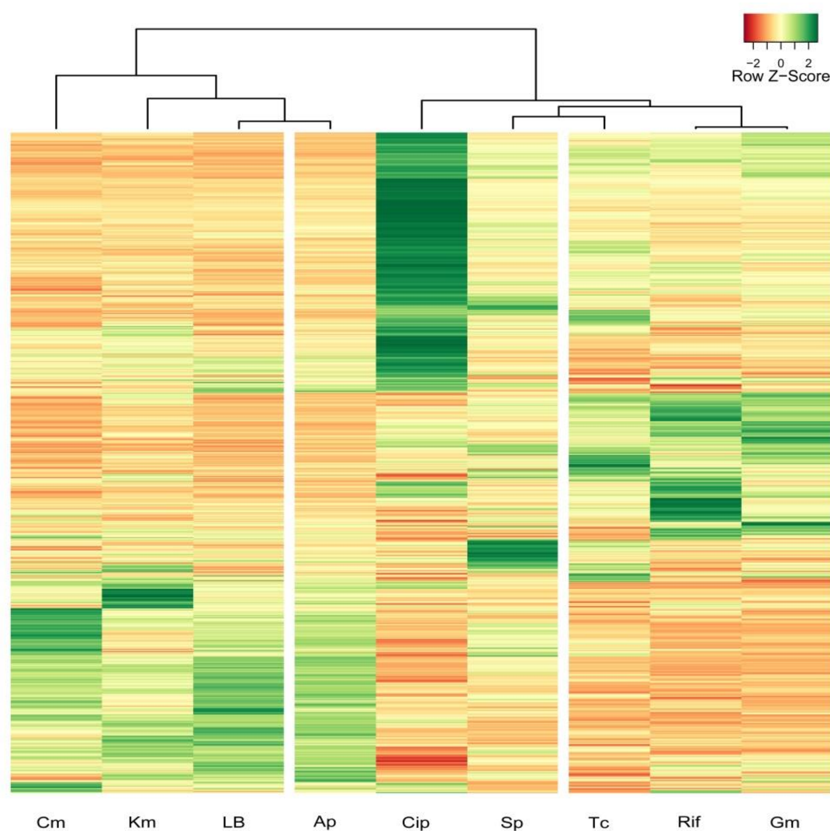

Figure 1. Heatmap and hierarchical cluster analysis of expression of the most differentially expressed mRNAs in the presence of antibiotics (Ap, Cm, Km, Tc, Cip, Gm, Sp and Rif) (*P*-value<0.05) and in the absence of antibiotics (LB). Green color represents mRNAs with high expression, and red color indicates mRNAs with low expression.

Functional composition of DOT-T1E transcriptome following exposure to antibiotics

Using the criteria of gene expression fold-change <-2 and >2 and *p*-values <0.02, we identified genes expressed differentially in the presence of each of the antibiotics tested. Based on the analysis of the heatmaps, the antibiotics were clustered in two groups. One group had less differences among them when compared with the control conditions, these included ampicillin, chloramphenicol and kanamycin. The second group, which consisted on ciprofloxacin, tetracycline, rifampicin, spectinomycin and gentamicin showed similar patterns but were different from the first three antibiotics. We did not identify common up-regulated genes when we compared the expression pattern in response to ampicillin, chloramphenicol and kanamycin (Figure 2A). However, we found several genes which were commonly up-regulated when we compared the expression pattern of two conditions (23 genes in the case of kanamycin-ampicillin, 5 genes between kanamycin and chloramphenicol and 3 genes between ampicillin and chloramphenicol) (Table S2).

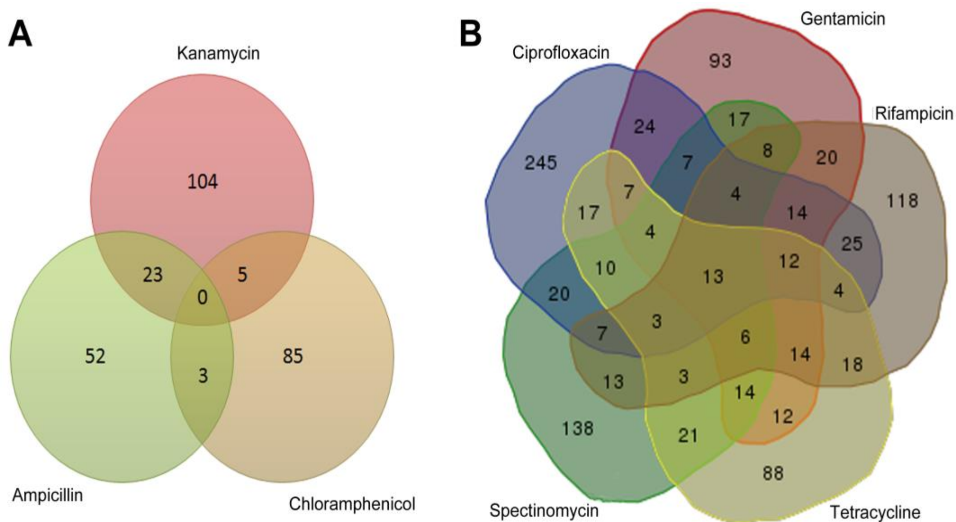

Figure 2. Venn diagrams representing the mRNAs up-regulated (two-fold change) in the presence of antibiotics compared with the control condition (*P*-value<0.02); A) Ampicillin (green), chloramphenicol (brown) and kanamycin (red); B) ciprofloxacin (blue), rifampicin (brown), tetracycline (yellow), spectinomycin (green) and gentamicin (red).

The expression pattern in the other five conditions assayed (ciprofloxacin, tetracycline, rifampicin, spectinomycin and gentamicin) (Figure 2B) showed that many genes appeared to be up-regulated in response to two, three and four antibiotics, while 13 genes (T1E_0241, T1E_0387, T1E_0388, T1E_1207, T1E_1232, T1E_2541, T1E_2652, T1E_2968, T1E_4162, T1E_4442, T1E_5244, T1E_5765, and T1E_5848) were up-regulated in the presence of all five antimicrobial conditions (Table S3). Of these 13 up-regulated genes, most belonged to functional groups that include tRNA related genes (Val and Gln tRNAs), efflux pumps (transporters and the *ttgC* gene from TtgABC efflux pump) and transcriptional regulators (LysR and a heavy metal sensor). The up-regulation of these genes clearly shows the importance of efflux pumps in the defense of bacteria against antimicrobials and the up-regulation of tRNAs demonstrates that these antibiotics interfere with the synthesis of proteins.

In the detailed functional analysis based on Gene Ontology (GO), the most represented functional categories at the transcriptional level in all conditions were related to binding (referring to the binding of ions, amines, cofactors, lipids, proteins, etc.), catalytic activities (involving activities such as lyase, isomerase, hydrolase, catalase, transferase, ligase, etc.) and metabolic processes (involving functions related to catabolism, biosynthesis of cellular components, iron-sulfur cluster assembly, nitrogen metabolism or methylation among others) (Figure 3). The majority of functional categories were similarly represented in all the antibiotic conditions tested but we found some differences and exceptions. For instance, there are GO categories that are only over-represented in the presence of a few conditions, i.e., developmental processes (up-regulated in the presence of ampicillin, ciprofloxacin, spectinomycin and tetracycline conditions), locomotion (not up-regulated in the presence of ampicillin, chloramphenicol and spectinomycin), translation regulatory activity (not up-regulated in the presence of rifampicin, chloramphenicol and kanamycin) or electron carrier activity (not up-regulated the presence of ampicillin and kanamycin). Other GO groups showed a wide variation in the percentage of genes up- and down-regulated, such as catalytic activity (from 22% in the presence of chloramphenicol to 42% with ciprofloxacin), ribosome proteins (from 1.3% in the presence of ampicillin to 24% in chloramphenicol), and membrane (5.6% in chloramphenicol to 17.6% in gentamicin) (Figure 3).

These results are in concordance with previous studies of the transcriptome of *P. putida* using DNA microarrays in response to ampicillin and chloramphenicol (Fernández *et al.*, 2012; Molina-Santiago *et al.*, 2014a). Overall, RNA-seq and DNA microarrays revealed that gene expression of *P. putida* KT2440 and DOT-T1E in the presence of chloramphenicol and ampicillin are different. These results are in agreement with those of gene expression of *S. coelicolor* and *M. tuberculosis* in the presence of ciprofloxacin and rifampicin, respectively (de Knegt *et al.*, 2013; Patkari and Mehra, 2013).

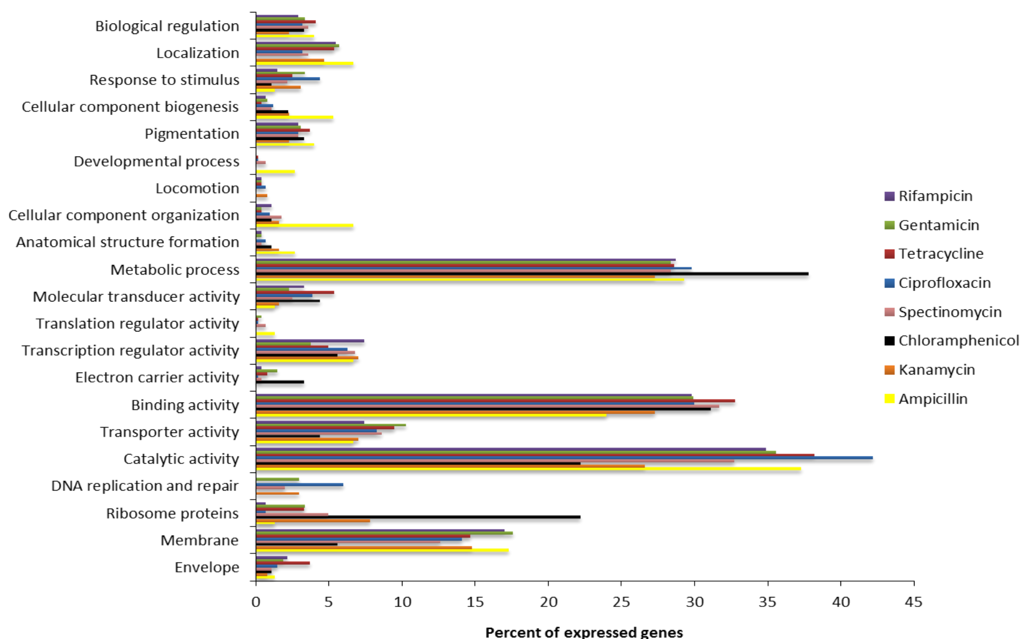

Figure 3. Classification of genes up-regulated (two-fold change) in the presence of antibiotics compared with the control condition (*P*-value<0.02) according to Gene Ontology (GO) classification. Main GO groups are represented for each condition in percent of expressed genes of each GO group compared with the total of genes expressed for each condition tested.

In the presence of chloramphenicol cells exhibited up- and down-regulation of genes involved in protein biosynthesis, nutrient transport, gene regulation, and stress and metabolism, while the set of genes induced and repressed by ampicillin are involved in amino acid metabolism, protein secretion, DNA metabolism, transcriptional regulation, organic ion metabolism, energy production, and membrane proteins (Fernández *et al.*, 2012; Molina-Santiago *et al.*, 2014a). Previous studies on the response to ciprofloxacin by *S. coelicolor* and our current results in

DOT-T1E revealed that, in response to this antibiotic, genes encoding proteins involved in DNA repair and recombination together with genes related to metabolism, transport, transcriptional regulation are up-regulated. Studies in the presence of rifampicin in *M. tuberculosis* showed up-regulated functional groups related to lipid metabolism, regulatory proteins, cell processes, intermediary metabolism and respiration (de Knegt *et al.*, 2013; Patkari and Mehra, 2013).

These analyses confirm that antibiotics induce a general stress response in *P. putida* DOT-T1E, changing the expression of various gene functions, but it also shows that the up-regulation of some functional groups is motivated by the requirement to counteract the mechanism of action of the encountered antibiotic.

mRNA and tRNA transcriptomic profiles agree with the different mechanism of action of the distinct antibiotics

Despite the fact that the GO categories were similar in all conditions assayed, when specific analyses of mRNA and tRNA were performed, we realized that within each GO category different genes were up- or down-regulated for each antibiotic tested. This clearly indicates the different mechanism of action of antibiotics and the different defense mechanisms of the DOT-T1E strain to compounds tested. Of note, a high percentage of the up-regulated genes were hypothetical proteins, what suggests that our knowledge of the cellular functions involved in antibiotic resistance is far from being fully understood.

Some functions were up-regulated in most of the conditions tested. For instance, glutathione metabolism, which has been indirectly related to antibiotic resistance (Cameron and Pakrasi, 2011; Molina-Santiago *et al.*, 2014a), appeared to be up-regulated in the presence of all antibiotics (T1E_3090; T1E_0524; T1E_3573; T1E_0339; T1E_2391) confirming that glutathione is an important molecule for antibiotic resistance in *P. putida* DOT-T1E (Tables S4-S11). Thiamine and folate biosynthesis could also be indirectly related with antibiotic resistance as reported by Eudes *et al.* (2008), where production of folate in *Bacillus anthracis* or *Clostridium botulinum* conferred intrinsic antibiotic resistance and agrees with the fact that certain antibiotics have been reported to inhibit folic acid synthesis (Kohanski *et al.*, 2010). DOT-T1E showed up-regulation of genes involved in the biosynthesis of these compounds (T1E_2161, T1E_4666; T1E_0866; T1E_3544) in the presence of the antibiotics tested, supporting the role of folate and thiamine in antimicrobial resistance. Different efflux pumps and

transporters, lipid and membrane related genes, transcriptional regulators and genes involved in energy production appeared to be up-regulated under the pressure of each antibiotic, confirming that the presence of these drugs activated general defense mechanisms (Tables S4-S11).

When we focused the study on mRNA and tRNA expression in each specific condition, transcriptomic analysis of DOT-T1E in the presence of chloramphenicol confirmed that it acts by binding to the 50S ribosome subunit inhibiting peptide bond formation and protein synthesis (Hahn *et al.*, 1955; Wolfe and Hahn, 1965). Up- and down-regulation of 50S ribosomal proteins were found together with a strong down-regulation of aminoacyl-tRNAs (Met-tRNA and Leu-tRNA) and the inhibition of some chaperone proteins involved in protein folding (Table S4).

The mRNA and tRNA expression profiles of kanamycin, tetracycline, gentamicin and spectinomycin showed regulation of 30S ribosomal proteins and a strong down-regulation of tRNA genes (Weinstein *et al.*, 1967; Pestka, 1975; Misumi and Tanaka, 1980; Connell *et al.*, 2003; Kehrenberg and Schwarz, 2007; Zakeri and Wright, 2008; Kang *et al.*, 2012). Spectinomycin and gentamicin also showed down-regulation of chaperones; this may be related to the inhibition of protein synthesis in DOT-T1E. Kanamycin, gentamicin and spectinomycin, led to up- and down-regulation of genes involved in DNA replication and DNA repair, which could indicate a bactericidal action of these antibiotics in the DOT-T1E strain. Kanamycin and gentamicin, for example, clearly reduced the expression of T1E_4186; an ATPase involved in DNA repair, and also repressed expression of T1E_0219, a protein involved in mismatch repair. Spectinomycin induced genes involved in DNA recombination mechanisms (T1E_0733, T1E_3483), which could explain its bactericidal effect on DOT-T1E at the concentration tested. In this sense, tetracycline, in line with its role as a bacteriostatic antibiotic, did not influence the expression of DNA replication genes in *P. putida* DOT-T1E (Tables S5-S8).

Transcriptomic analysis of cells grown in the presence of ciprofloxacin indicated that a higher number of genes were up- and down-regulated than observed with the rest of antibiotics; this finding correlates with the strong antibiotic effects that this compound exerts against *P. putida* DOT-T1E (Table S9). Genes involved in DNA replication and repair were found to be highly altered in expression, a finding which can be explained by the fact that ciprofloxacin acts by regulating the expression of DNA gyrase and topoisomerase

(Drlica and Zhao, 1997; Pommier *et al.*, 2010). Other genes involved in replication and repair of DNA were up-regulated, clearly showing that cells are suffering an attack and in consequence, trigger the defense response against this antibiotic.

Assays performed in the presence of rifampicin revealed the down-regulation of DNA-directed RNA polymerase subunits and induction of RpoT, an alternative RNA-polymerase sigma-70 factor (Duque *et al.*, 2007). The inhibition of aminoacyl-tRNA genes explained, in part, the RNA synthesis suppression and consequently, cell death produced by the action of this bactericidal compound (Table S10).

Finally, transcriptomic expression obtained in the presence of ampicillin showed an induction of only one cell wall hydrolase gene (T1E_2302) related with the mechanism of action of this antimicrobial. Transcriptional regulators, efflux pumps and transporters were expressed but no penicillin binding proteins (PBPs) or autolysins related to the activity of ampicillin were induced; this could be explained by the fact that only a small number of genes were up-regulated in cells exposed to low ampicillin doses (Table S11).

Specific sRNA transcriptional patterns associated with antibiotic exposure

The most important regulatory mechanisms of gene expression are the involvement of transcriptional regulators that are able to regulate different genes or cascades of genes and thus activate or repress many cellular functions (Tropel and van der Meer, 2004; Maddocks and Oyston, 2008; Herrera *et al.*, 2012; Daddaoua *et al.*, 2013; Molina-Santiago *et al.*, 2014b). In recent years, sRNAs have been recognized as molecules with a role in fine-tunning gene regulation, affecting one or more of the following events: translation initiation, transcription termination, mRNA stability, and the activity of proteins that regulate gene expression due to short and imperfect base-pairing interactions with mRNAs (Levine *et al.*, 2007; Waters and Storz, 2009; Storz *et al.*, 2011). We hypothesized that sRNAs could play a role in the antibiotic resistance profiles obtained in DOT-T1E. Our transcriptomic analyses revealed the presence of 154 *trans*-encoded RNAs in the *P. putida* DOT-T1E genome (Table 1), of which only 16 were already described. We first studied the sequence conservation of these *trans*-encoded RNAs with respect to those described in other bacteria using the BLASTN algorithm. The sequences of 19.5% of the *trans*-encoded RNAs were not found in any other bacteria, and 6.5% were found to be conserved in

organisms outside the Pseudomonadales order, with homologous in Bacillales, Clostridiales, Oceanospirillales, Actinomycetales, Burkholderiales, Enterobacteriales, Chromatiales and Thiotrichales. For the rest of sRNAs their sequences matched at > 90% with sequences previously identified in other species of the genus *Pseudomonas*.

Different functions and characteristics were predicted for the DOT-T1E 16 sRNAs that have been previously described. Four of the already known sRNAs play roles in iron storage (*prrF*1 and *prrF*2), regulation of GacA/GacS activity (*rsmY*) or cleaving off sequences of RNA into tRNA molecules (RnaseP). Of the 138 novel sRNAs, 13 of them presented homologies with other previously described sRNAs in bacteria, including one CrcY and one CrcZ (carbohydrate metabolism regulation), which functions are known in KT2440, 3 cobalamin riboswitches that are involved in repression of the expression of proteins for vitamin B12 biosynthesis, 1 TPP riboswitch that binds directly to thiamine pyrophosphate to regulate gene expression, 1 FMN riboswitch that modulates flavin mononucleotide biosynthesis and transport proteins, 1 spot42 that is a regulator in carbohydrate metabolism and uptake whose expression is activated by glucose and inhibited by the cAMP-CRP complex, and 3 C4 (unknown function) present in many *Pseudomonas* strains.

We next looked at the conservation of sRNAs across 9 different *P. putida* strains and we observed that only 33% of all sRNAs detected in *P. putida* DOT-T1E were present in all the other genomes tested, suggesting a limited conservation and a rapid propagation between strains as found in *Pseudomonas aeruginosa* strains (Gomez-Lozano *et al.*, 2012). Central metabolism and general regulation functions are likely to be under the regulatory influence of the 51 sRNAs present in the core-genome of *P. putida* strains, although further studies are needed to substantiate their functions. For the rest of sRNAs detected that do not belong to the core-genome, we suggest that they could control specific functions of the strains tested. This could be the case for 41 sRNAs that are only present in *P. putida* DOT-T1E, F1 and Idaho strains; three solvent tolerant microorganisms.

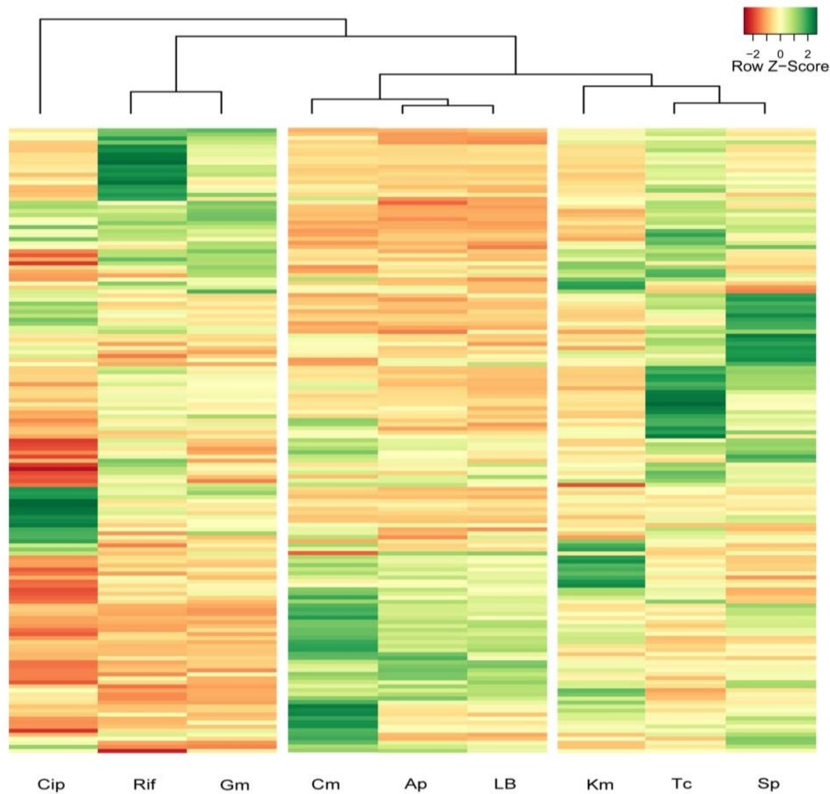

Figure 4. Heatmap and hierarchical cluster analysis of sRNA expression in the presence of antibiotics (Ap, Cm, Km, Tc, Cip, Gm, Sp and Rif) and in the absence of antibiotics (LB). Green color represents sRNAs with high expression, and red color indicates sRNAs with low expression.

When we analyzed the sRNA heatmap (Figure 4), the patterns of expression obtained for the 154 sRNAs showed similarities with those obtained for the mRNA, although it should be noted that this represents all 154 sRNAs detected versus the 604 most differentially expressed mRNAs. As for the mRNA analysis, the sRNA profiles obtained in cells exposed to ampicillin and chloramphenicol were the most similar to the control condition (LB), with a small difference in up-regulated genes in the case of chloramphenicol. The kanamycin map showed many expressed regions and a profile similar to the control condition, but with a highlighted region of up-regulated genes, similar to what was observed in the mRNA profile. Ciprofloxacin showed a pattern with a group of up-regulated sRNAs and an important group of strongly down-regulated sRNAs, in this case, the profiles of up- and down-regulated sRNAs were the opposite to what was

observed in the mRNA pattern; this indicates a regulation mechanism where an inhibition of sRNAs could be provoking an induction of mRNAs. Gentamicin and rifampicin showed two expression patterns very different to those obtained with the rest of the conditions tested. Both antibiotics showed similar expression patterns between them with the exception of a group of up-regulated sRNAs in the presence of rifampicin, a group that had previously been shown to be up-regulated in mRNA heatmaps (Figure 4).

Spectinomycin and tetracycline showed unique and different patterns with clear groups of up-regulated sRNAs, similar to these obtained from the mRNA heat maps, and in both cases, different to the profiles obtained with the other antimicrobial compounds. Only 14 of 154 sRNAs analysed were not expressed in at least one of the conditions tested. In the presence of ciprofloxacin, rifampicin, gentamicin and tetracycline there was a big number of sRNAs that were highly expressed (93, 81, 79 and 74 respectively). In contrast, ampicillin, kanamycin and chloramphenicol were the conditions with less up- and down-regulated sRNAs (6, 28 and 35 respectively) confirming that many sRNAs respond in a non-specific way to environmental stresses and that DOT-T1E harbours an important group of sRNAs with a specific response against each antibiotic tested (Table 1).

Taken together the mRNA and sRNA expression data suggest a potential regulatory role of sRNAs in antibiotic resistance. Further studies will be required to identify the action and role of specific sRNAs in antibiotic resistance.

Table 1. Intergenic sRNAs candidates detected in *P. putida* DOT-T1E.

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA1	4240	4383	144	-	<<>	T1E_0005	T1E_0006	-1.16	-1.03	1.21	1.25	1.75	1.05	1.37	1.21	++	-
RNA2	4360	4490	131	+	<>>	T1E_0005	T1E_0006	-1.18	-1.05	1.31	-1.54	0.47	0.22	0.18	0.20	++	-
RNA3	21510	21837	328	-	><<	T1E_0025	T1E_0026	-1.55	-1.35	-1.06	0.33	-1.17	2.63	4.08	6.61	+	CrcY
RNA4	88451	88626	176	+	<><	T1E_0078	T1E_0079	-1.12	1.93	1.99	-1.52	3.81	-1.19	4.95	-1.53	+	-
RNA5	132597	132693	97	+	>>>	T1E_0116	T1E_0117	-1.42	-1.38	-1.07	2.07	2.53	1.21	1.52	1.47	+	-
RNA6	134229	134447	219	-	><<	T1E_0117	T1E_0118	1.09	1.01	-1.32	0.49	-1.30	-1.75	-1.69	-1.21	+	-
RNA7	210270	210371	102	-	><>	T1E_0188	T1E_0189	1.01	-1.15	1.38	1.80	2.05	1.15	1.27	1.42	+	-
RNA8	445820	445995	176	+	<><	T1E_0395	T1E_0396	1.02	1.23	-1.46	0.24	1.28	-1.54	-1.06	1.07	+	-
RNA9	499992	500127	136	-	<<<	T1E_0438	T1E_0439	1.12	1.31	-1.07	0.30	1.35	-1.95	1.25	-1.23	+	-
RNA10	607269	607411	143	+	<>>	T1E_0535	T1E_0536	1.07	-1.10	1.37	1.03	-1.01	1.35	1.34	1.17	+	-
RNA11	609283	609482	200	-	<<>	T1E_0537	T1E_0538	1.05	1.39	1.82	3.44	1.78	3.54	3.27	3.15	+	-
RNA12	658606	658716	111	+	>><	T1E_0580	T1E_0581	1.22	1.32	-1.29	0.25	1.57	-1.43	1.50	1.19	+	-
RNA13	659181	659274	94	+	<>>	T1E_0581	T1E_0582	-1.04	1.03	1.09	0.24	2.06	1.81	6.13	2.24	+	-
RNA14	659519	659693	175	+	<>>	T1E_0581	T1E_0582	1.03	1.44	1.69	0.11	0.40	0.32	-1.94	0.29	+	C4
RNA15	674650	674797	148	+	<>>	T1E_0597	T1E_0598	-1.16	-1.31	1.50	0.36	-1.41	-1.36	-1.11	-1.28	+	-
RNA16	702259	702602	344	-	><>	T1E_0622	T1E_0623	-1.19	1.09	-1.23	0.44	-1.02	3.21	3.79	7.63	+	CrcZ
RNA17	744859	744985	127	-	<<>	T1E_0658	T1E_0659	1.42	1.23	1.11	-1.20	2.03	2.85	3.71	2.27	+	-
RNA18	833297	833398	102	-	<<<	T1E_0733	T1E_0734	-1.10	1.42	-1.06	0.46	0.47	0.31	0.39	0.39	+	-
RNA19	837422	837557	136	+	<>>	T1E_0737	T1E_0738	1.07	1.94	1.98	2.01	2.01	2.82	2.17	3.89	+	C4

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA20	967240	967377	138	+	<><	T1E_0850	T1E_0851	-1.26	0.36	-1.43	-1.33	1.31	0.39	-1.06	0.39	+	-
RNA21	967454	967595	142	+	<><	T1E_0850	T1E_0851	1.24	1.66	2.22	11.05	8.51	8.33	6.36	7.97	+	-
RNA22	967498	967595	98	-	<<<	T1E_0850	T1E_0851	-1.24	1.17	1.26	1.82	2.14	2.82	2.33	2.40	+	-
RNA23	1060943	1061038	96	+	>>>	T1E_0940	T1E_0941	-1.20	-1.18	1.24	6.19	2.02	-1.12	1.45	2.74	++	-
RNA24	1169911	1170073	163	+	<>>	T1E_1026	T1E_1027	1.19	1.31	1.23	-1.70	-1.29	-1.03	1.09	-1.06	++	-
RNA25	1254202	1254326	125	-	<<<	T1E_1099	T1E_1100	1.17	2.83	1.22	0.17	1.24	-1.95	1.58	-1.90	+	-
RNA26	1362768	1362977	210	-	<<>	T1E_1188	T1E_1189	-1.36	-1.47	-1.26	0.26	-1.33	-1.38	1.13	1.11	+	TPP
RNA27	1402669	1402763	95	-	><<	T1E_1220	T1E_1221	-1.04	0.22	-1.76	-1.18	-1.41	-1.30	0.48	0.44	+	-
RNA28	1436622	1436699	78	-	><>	T1E_1248	T1E_1249	1.20	1.16	-1.43	0.34	-1.23	-1.31	-1.48	0.36	+	-
RNA29	1480766	1481018	253	+	>><	T1E_1287	T1E_1288	1.15	1.08	-1.29	2.26	-1.66	1.03	-1.67	-1.49	+	-
RNA30	1480787	1481052	266	-	><<	T1E_1287	T1E_1288	-1.01	-1.15	-1.09	6.64	3.71	2.89	2.93	2.22	+	-
RNA31	1491872	1492157	286	+	>><	T1E_1300	T1E_1302	1.18	1.21	-1.07	-1.04	-1.02	-1.13	-1.05	0.44	+	-
RNA32	1500427	1500738	312	-	<<>	T1E_1312	T1E_1313	1.30	1.51	1.32	1.30	2.32	1.77	4.15	1.95	---	-
RNA33	1512295	1512421	127	+	<>>	T1E_1331	T1E_1132	-1.49	3.18	1.99	-1.77	1.10	-1.89	-1.63	0.37	+	-
RNA34	1514704	1515258	555	+	<><	T1E_1337	T1E_1338	-1.29	2.96	1.46	0.46	1.21	-1.60	-1.07	0.45	---	-
RNA35	1522624	1522830	207	-	><>	T1E_1351	T1E_1352	1.36	-1.04	3.22	3.06	3.70	4.36	5.38	2.67	---	-
RNA36	1541024	1541233	210	+	>>>	T1E_1378	T1E_1379	-1.22	1.17	-1.22	0.17	-1.15	0.31	0.35	0.21	---	-
RNA37	1549103	1549245	143	-	><<	T1E_1385	T1E_1386	1.09	1.06	1.20	0.46	1.09	-1.74	1.30	-1.07	---	-
RNA38	1553918	1554133	216	-	<<<	T1E_1395	T1E_1396	1.10	1.37	-1.20	0.11	-1.19	-1.86	-1.72	0.28	---	-
RNA39	1566959	1567089	131	-	<<<	T1E_1410	T1E_1411	1.79	1.88	1.66	1.71	3.21	1.69	2.56	1.05	---	-
RNA40	1574044	1574276	233	-	><>	T1E_1422	T1E_1423	1.10	1.22	2.00	5.36	6.38	2.29	4.31	1.42	+	-

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA41	1574830	1574988	159	-	><>	T1E_1422	T1E_1423	1.06	3.27	1.61	0.29	1.91	1.49	1.45	-1.67	---	-
RNA42	1604665	1604747	83	+	>>>	T1E_1454	T1E_1455	-1.19	1.45	-1.01	0.27	-1.73	-1.66	-1.17	-1.33	---	-
RNA43	1606933	1607157	225	-	><>	T1E_1458	T1E_1459	2.02	4.54	1.96	1.11	3.23	3.31	5.37	2.55	+	-
RNA44	1658471	1658563	93	+	>>>	T1E_1502	T1E_1503	-1.35	1.43	0.40	0.10	0.41	0.13	0.18	0.10	---	-
RNA45	1718067	1718179	113	+	>>>	T1E_1555	T1E_1556	-1.24	1.37	1.37	8.06	5.27	10.26	6.84	8.00	+	-
RNA46	1927167	1927300	134	-	><>	T1E_1757	T1E_1758	1.14	-1.01	1.43	-1.26	1.35	1.54	1.83	-1.24	+	-
RNA47	1934495	1934598	104	-	><>	T1E_1764	T1E_1765	-1.22	2.19	1.28	0.18	1.57	0.46	-1.11	0.36	++	-
RNA48	1996423	1996695	273	-	><<	T1E_1823	T1E_1824	-1.08	1.53	0.30	0.15	-1.91	0.17	0.25	0.09	+	-
RNA49	2191801	2191936	136	-	><<	T1E_2001	T1E_2002	-1.01	1.69	-1.08	6.24	6.85	8.70	7.03	5.84	---	-
RNA50	2375714	2375838	125	+	>>>	T1E_2170	T1E_2171	1.00	1.70	1.11	0.23	1.23	1.18	1.03	-1.15	+	-
RNA51	2380964	2381132	169	-	><>	T1E_2173	T1E_2174	1.20	2.67	1.85	1.72	10.90	4.51	14.79	8.29	+	-
RNA52	2404652	2404913	262	-	<<>	T1E_2196	T1E_2197	1.20	1.25	-1.35	0.18	0.49	0.43	1.33	-1.70	+	-
RNA53	2471588	2471683	96	+	<>>	T1E_2261	T1E_2262	1.02	-1.12	1.17	-1.65	-1.47	0.41	0.44	0.31	+	-
RNA54	2485291	2485468	178	-	><>	T1E_2275	T1E_2276	1.42	1.36	2.10	1.06	-1.20	1.51	1.09	1.54	+	-
RNA55	2563455	2563587	133	+	<><	T1E_2344	T1E_2345	1.28	-1.01	2.13	2.98	2.21	6.93	5.97	13.75	---	-
RNA56	2569661	2569856	196	+	<>>	T1E_2347	T1E_2348	-1.25	0.41	-1.48	0.15	-1.35	0.41	1.12	-1.32	---	-
RNA57	2640427	2640578	152	+	>><	T1E_2408	T1E_2409	1.17	1.18	1.97	8.80	1.72	2.15	1.73	1.05	+	-
RNA58	2723898	2724184	287	+	<><	T1E_2481	T1E_2482	-1.13	2.14	1.27	0.50	1.66	1.05	2.48	1.19	+	-
RNA59	2746619	2746814	196	-	><<	T1E_2503	T1E_2504	-1.24	-1.14	-1.63	-1.40	1.67	1.96	2.90	10.81	+	-
RNA60	2766823	2767154	332	+	<><	T1E_2515	T1E_2516	1.52	1.78	-1.15	0.22	3.07	1.93	5.72	1.98	---	-
RNA61	2767551	2767643	93	-	<<<	T1E_2515	T1E_2516	1.17	-1.26	-1.18	0.25	-1.34	0.28	-1.49	0.45	+	-

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA62	2770326	2770476	151	-	<<>	T1E_2519	T1E_2520	1.10	1.44	1.98	0.26	-1.93	-1.38	-1.43	-1.55	+	-
RNA63	2865165	2865269	105	+	<>>	T1E_2616	T1E_2617	1.24	3.59	2.08	1.06	1.87	-1.13	1.58	1.78	+	-
RNA64	2877857	2878062	206	+	>>>	T1E_2629	T1E_2630	1.23	2.25	-1.23	0.28	2.20	0.45	1.77	1.17	+	-
RNA65	3005217	3005352	136	+	>><	T1E_2755	T1E_2756	1.19	2.55	2.09	1.10	3.72	2.73	4.86	7.10	+	-
RNA66	3054421	3054569	149	+	>>>	T1E_2798	T1E_2799	1.23	1.32	1.40	0.21	-1.05	-1.04	3.63	-1.52	+	-
RNA67	3067976	3068102	127	+	<>>	T1E_2810	T1E_2811	-1.16	2.90	2.28	1.57	2.71	1.76	2.45	4.28	+	-
RNA68	3089088	3089163	76	-	><<	T1E_2826	T1E_2827	-1.45	0.50	1.21	-1.87	-1.46	1.26	1.50	-1.37	+	-
RNA69	3120495	3120636	142	+	>>>	T1E_2855	T1E_2856	-1.07	2.07	1.31	1.34	1.87	1.09	1.01	1.32	+	-
RNA70	3120529	3120603	75	-	><>	T1E_2855	T1E_2856	-1.17	1.16	1.91	3.71	6.48	3.45	4.49	4.10	+	-
RNA71	3138595	3138690	96	-	<<>	T1E_2872	T1E_2873	1.57	1.38	2.09	1.43	3.41	3.65	10.27	3.76	++	-
RNA72	3158437	3158920	484	+	<>>	T1E_2889	T1E_2890	1.02	3.28	1.53	0.40	4.58	2.70	5.91	3.61	---	-
RNA73	3208054	3208160	107	-	><>	T1E_2925	T1E_2926	-1.03	1.30	-1.07	0.24	0.44	0.46	0.37	0.50	+	-
RNA74	3213830	3213930	101	+	>>>	T1E_2932	T1E_2933	1.09	-1.22	-1.16	0.45	1.06	0.26	0.49	0.14	+	-
RNA75	3241534	3241668	135	-	><<	T1E_5807	T1E_2963	1.06	1.08	1.07	0.37	1.11	-1.00	1.29	1.23	+	-
RNA76	3333572	3333746	175	-	<<<	T1E_3042	T1E_3043	1.57	3.87	1.64	2.14	9.37	1.61	4.33	1.69	---	-
RNA77	3346526	3346642	117	+	<><	T1E_3050	T1E_3051	1.26	1.30	1.06	1.21	3.17	2.08	4.37	1.82	---	-
RNA78	3413578	3413821	244	+	<><	T1E_3112	T1E_3113	-1.02	-1.17	-1.36	-1.59	1.76	2.81	2.21	4.49	+	-
RNA79	3534013	3534179	167	+	>>>	T1E_3215	T1E_3216	1.02	1.24	-1.71	0.09	-1.18	0.08	0.36	0.13	+	-
RNA80	3540788	3540943	156	+	>><	T1E_3221	T1E_3222	1.74	3.89	2.26	2.45	10.72	5.91	15.46	6.49	---	-
RNA81	3541176	3541279	104	+	>><	T1E_3221	T1E_3222	1.36	2.64	-1.07	1.34	3.43	2.19	4.36	1.91	++	-
RNA82	3544624	3544770	147	-	><>	T1E_3223	T1E_5808	1.74	1.86	0.31	0.21	-1.33	0.33	-1.17	0.27	---	-

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA83	3567518	3567629	112	+	<><	T1E_3243	T1E_3244	1.07	1.46	1.42	1.91	1.11	1.42	1.63	1.36	+	-
RNA84	3567926	3568255	330	-	<<<	T1E_3243	T1E_3244	1.23	-1.36	1.09	0.47	-1.95	3.08	1.10	4.11	+	rmf
RNA85	3619100	3619323	224	+	>>>	T1E_3283	T1E_3284	-1.03	1.49	-1.35	0.39	1.09	0.23	-1.90	0.27	+	-
RNA86	3619456	3619633	178	+	>>>	T1E_3283	T1E_3284	1.40	2.35	-1.58	0.16	1.26	0.38	-1.06	0.30	+	-
RNA87	3739093	3739323	231	+	>><	T1E_3369	T1E_3370	1.10	1.78	-1.81	0.40	1.21	0.12	-1.41	0.21	+	-
RNA88	3753667	3753813	147	+	>>>	T1E_3384	T1E_3385	1.70	3.83	-1.31	3.57	5.35	24.53	31.07	97.97	+	-
RNA89	3771889	3772004	116	+	<><	T1E_3403	T1E_3404	1.40	1.40	0.16	0.31	1.11	1.32	1.30	1.25	+	-
RNA90	3885234	3885393	160	+	<><	T1E_3540	T1E_3541	-1.02	1.22	1.42	0.38	-1.20	-1.48	-1.04	0.50	+	-
RNA91	4011521	4011705	185	+	<><	T1E_3650	T1E_3651	1.04	1.22	1.55	1.20	1.69	1.64	1.64	1.86	+	Cobalamin
RNA92	4134780	4134928	149	+	>>>	T1E_3768	T1E_3770	-1.01	-1.44	-1.72	0.05	0.48	0.32	0.47	0.25	+	-
RNA93	4154465	4154871	407	+	>><	T1E_3790	T1E_3791	1.16	1.85	0.47	0.32	1.50	0.13	-1.81	0.17	+	-
RNA94	4256285	4256554	270	-	<<>	T1E_3885	T1E_3886	-1.26	-1.45	-1.03	1.16	1.62	-1.19	1.35	-1.16	+	-
RNA95	4279540	4279631	92	-	<<>	T1E_3908	T1E_3909	-1.30	1.52	1.85	1.32	-1.44	-1.03	1.01	0.45	+	-
RNA96	4327612	4327691	80	-	><<	T1E_3958	T1E_3959	1.20	1.08	1.62	0.47	0.39	-1.88	-1.42	-1.07	+	-
RNA97	4389519	4389709	191	-	><<	T1E_4016	T1E_4017	-1.08	-1.30	-1.28	5.24	9.64	1.61	3.47	3.17	+	-
RNA98	4389568	4389876	309	+	>><	T1E_4016	T1E_4017	1.11	1.16	1.83	6.65	3.50	2.54	3.08	1.89	+	-
RNA99	4403327	4403430	104	+	<><	T1E_4032	T1E_4033	1.20	1.20	1.53	1.37	-1.17	1.71	1.03	1.21	+	-
RNA100	4407429	4407528	100	-	><>	T1E_4036	T1E_4037	2.26	1.73	1.80	1.41	2.36	2.03	5.72	3.04	+	-
RNA101	4431385	4431476	92	-	><>	T1E_4058	T1E_4059	1.13	1.46	1.32	0.30	-1.70	1.02	1.03	-1.45	+	-
RNA102	4442919	4443247	329	+	<><	T1E_4063	T1E_4064	1.01	1.13	1.29	1.18	1.63	-1.11	1.30	-1.05	+	-
RNA103	4562773	4562884	112	-	><>	T1E_4183	T1E_4184	1.14	1.06	2.04	2.62	3.20	4.06	3.85	4.33	+	-

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA104	4572647	4572767	121	+	>>>	T1E_4188	T1E_4189	2.34	2.25	-1.32	0.19	0.30	0.42	0.45	-1.44	+	-
RNA105	4614007	4614124	118	+	<>>	T1E_4237	T1E_4238	-1.05	1.60	1.41	-1.40	-1.29	0.44	-1.40	0.45	+	-
RNA106	4796314	4796795	482	+	>>>	T1E_4408	T1E_4409	1.17	1.24	1.09	-1.02	1.39	-1.06	1.04	1.37	+	-
RNA107	4887502	4887720	219	+	>>>	T1E_4490	T1E_4491	-1.04	1.24	1.90	1.83	1.47	2.63	2.31	2.84	+	-
RNA108	4919939	4920077	139	+	<><	T1E_4513	T1E_4514	-1.02	2.41	1.42	1.48	7.68	2.76	9.27	6.53	+	-
RNA109	4957625	4957740	116	-	><<	T1E_4547	T1E_4548	1.26	2.35	2.75	1.05	1.73	1.92	2.40	1.75	---	-
RNA110	4959148	4959345	198	-	<<>	T1E_4548	T1E_4549	-1.04	-1.20	-1.55	0.15	-1.69	0.39	-1.74	0.29	---	-
RNA111	4979443	4979939	497	-	><>	T1E_4557	T1E_4558	1.33	1.34	1.83	0.26	-1.37	-1.04	1.00	-1.17	---	-
RNA112	5006832	5007194	363	+	<><	T1E_4578	T1E_4579	1.27	2.31	1.07	1.22	5.58	1.12	3.89	1.54	---	-
RNA113	5136270	5136535	266	+	>>>	T1E_4698	T1E_4699	2.02	5.73	2.48	8.87	25.16	13.14	31.40	74.54	+	-
RNA114	5137478	5137779	302	-	><>	T1E_4698	T1E_4699	1.40	2.68	-1.60	2.94	6.79	3.47	32.49	4.69	+	-
RNA115	5194796	5195043	248	+	<>>	T1E_4755	T1E_4756	1.11	1.06	-1.29	3.00	1.58	1.65	3.03	2.39	+	-
RNA116	5195899	5195975	77	+	>><	T1E_4756	T1E_4757	-1.27	-1.08	1.20	1.26	1.95	-1.36	1.10	0.42	+	-
RNA117	5196101	5196331	231	+	>><	T1E_4756	T1E_4757	1.10	1.68	1.48	2.59	3.38	6.19	5.96	11.16	+	Spot42
RNA118	5250482	5250676	195	-	><<	T1E_4805	T1E_4806	1.17	1.53	1.03	0.16	2.09	1.01	1.03	1.28	+	-
RNA119	5316113	5316197	85	+	>><	T1E_4867	T1E_4868	1.34	1.28	-1.06	-1.58	-1.55	0.30	0.38	0.19	---	-
RNA120	5353698	5353963	266	-	><>	T1E_4904	T1E_4905	1.13	1.27	-1.79	1.36	1.23	0.33	-1.38	0.34	---	-
RNA121	5358427	5358567	141	-	><>	T1E_4907	T1E_4908	-1.07	1.39	1.28	1.02	1.24	1.17	1.86	1.37	---	-
RNA122	5416204	5416320	117	-	><>	T1E_4963	T1E_4964	1.01	1.20	1.81	-1.12	1.28	2.31	2.41	2.74	+	-
RNA123	5434205	5434291	87	-	<<>	T1E_4982	T1E_4983	1.51	1.29	-1.52	9.30	1.38	-1.05	-1.35	1.22	+	-
RNA124	5474866	5475045	180	-	><>	T1E_5026	T1E_5027	1.17	1.18	1.05	13.44	16.50	3.25	3.21	2.44	+	-

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
RNA125	5491072	5491365	294	+	<><	T1E_5042	T1E_5043	1.31	2.13	-1.77	1.20	1.92	0.26	-1.03	0.34	---	-
RNA126	5493971	5494109	139	+	<><	T1E_5045	T1E_5046	1.13	1.40	2.33	0.30	-1.91	-1.77	-1.07	-1.43	+	C4
RNA127	5547818	5547893	76	+	>><	T1E_5102	T1E_5103	-1.47	1.15	-1.30	0.22	1.07	-1.26	1.56	1.22	+	-
RNA128	5566525	5566621	97	+	>><	T1E_5120	T1E_5121	1.10	1.10	1.89	3.38	1.67	2.15	1.37	2.24	+	-
RNA129	5654234	5654404	171	-	><>	T1E_5198	T1E_5199	1.04	-1.30	1.30	1.94	2.56	2.31	3.46	2.84	+	Cobalamin
RNA131	5697861	5697900	40	+	>><	T1E_5237	T1E_5238	-1.32	1.16	-1.19	1.63	-1.13	1.43	1.21	-1.48	+	-
RNA132	5722500	5722653	154	-	><>	T1E_5260	T1E_5261	-1.16	1.10	-1.31	1.19	1.66	-1.19	1.24	1.02	+	Cobalamin
RNA133	5769896	5770019	124	+	<><	T1E_5302	T1E_5303	1.73	-1.05	2.54	10.42	1.70	2.30	2.77	4.19	+	-
RNA134	5843591	5843791	201	+	<><	T1E_5368	T1E_5369	1.16	1.11	1.47	-1.37	1.15	1.54	1.64	1.50	+	-
RNA135	5963624	5963713	90	-	<<<	T1E_5471	T1E_5472	1.04	1.42	1.04	0.14	-1.12	0.38	-1.26	-1.69	---	-
RNA136	6024296	6024403	108	-	<<<	T1E_5525	T1E_5526	-1.16	-1.17	1.41	0.21	-1.28	1.27	-1.17	1.26	---	-
RNA137	6061596	6061938	343	+	>>>	T1E_5557	T1E_5558	-1.31	-1.15	-1.20	0.25	-1.04	0.44	-1.63	-1.84	---	-
RNA138	6244618	6244790	173	-	><<	T1E_5740	T1E_5741	-1.06	-1.10	1.97	0.16	-1.22	-1.16	-1.20	-1.61	---	-
RNA139	6248987	6249114	128	+	<>>	T1E_5745	T1E_5746	1.03	-1.32	1.59	1.46	2.90	5.88	4.96	16.51	++	FMN
prrF2	64952	65099	148	+	<><	T1E_0059	T1E_0060	1.53	5.28	72.56	21.32	4.91	26.50	16.15	56.04	+	prrF2
P9_1	125690	125763	74	+	>>>	T1E_0111	T1E_0112	1.00	5.50	14.00	10.50	1.00	1.00	1.00	1.00	+	P9_1
T1E_5771	838722	838821	100	+	>><	T1E_0738	T1E_0739	-1.32	1.37	1.47	1.91	1.31	2.10	1.90	1.49	+	-
P26	2121856	2121921	66	+	<><	T1E_1936	T1E_1937	1.05	-1.69	2.24	2.73	1.27	-1.06	1.39	-1.25	+	P26
prrF1	2321564	2321712	149	+	>><	T1E_2124	T1E_2125	-1.53	1.31	2.75	50.98	4.53	35.51	13.74	18.06	+	prrF1
rsmY	2389011	2389137	127	+	>><	T1E_2181	T1E_2182	-1.30	1.22	2.07	-1.78	1.25	2.40	2.00	2.07	+	rsmY
P15	2672175	2672293	119	+	<><	T1E_2437	T1E_2438	1.30	5.71	1.43	1.26	4.36	2.97	3.45	3.62	+	P15

Name	Start[a]	End[a]	Size[b]	Strand	Direction genes[c]	5' flanking gene	3' flanking gene	Fold change								blastn[d]	Rfam[e]
								Ap	Cm	Km	Cip	Sp	Gm	Tc	Rif		
t44	2816088	2816233	146	+	<>>	T1E_2564	T1E_2565	0.29	1.15	0.35	1.50	-1.15	0.46	-1.04	-1.34	+	t44
rnaseP	4726421	4726796	376	+	>>>	T1E_4346	T1E_4347	0.38	-1.78	-1.89	1.26	1.61	1.18	1.55	1.50	+	rnaseP
ssrS	4815246	4815425	180	+	<>>	T1E_4921	T1E_4922	1.14	1.62	2.00	5.75	1.82	3.82	2.48	2.97	+	ssrS
P9_2	4837632	4837811	180	+	<><	T1E_4423	T1E_4424	-1.02	-1.34	-1.10	1.48	1.31	1.72	2.49	1.88	+	P9_2
P24	5372006	5372259	254	+	<>>	T1E_4443	T1E_4444	-1.77	0.30	-1.23	3.26	2.82	1.53	3.09	12.63	+	P24
T1E_5829	5387379	5387450	72	+	>><	T1E_4937	T1E_4938	1.12	-1.34	1.03	-1.12	1.67	1.09	1.22	-1.19	---	-
T1E_5830	5388113	5388184	72	+	>><	T1E_4937	T1E_4938	1.61	1.42	2.48	2.36	3.78	3.22	3.39	1.94	---	-
prrB_rsmZ	5426035	5426197	163	+	>><	T1E_4974	T1E_4975	1.07	2.19	1.90	5.85	8.74	1.82	6.06	1.92	+	prrB_rsmZ
P16	5462917	5463111	195	+	>>>	T1E_5017	T1E_5018	1.55	1.87	1.67	1.07	2.10	2.62	4.05	4.95	+	P16

a The coordinates of the transcript predicted by RNA sequencing data.

b Length of the sRNA candidate predicted by RNA sequencing data.

c The middle arrow indicates the orientation of the sRNA, while the flanking arrows indicate the orientation of the adjacent genes.

d The sequence conservation of candidate sRNAs in other microbial organisms was investigated using the BLASTN algorithm. (---), no sequence conservation found; (+) sequence conserved primarily in pseudomonadaceae; (++) sequence conserved in bacterial species outside the pseudomonodaceae family.

e Candidate sRNAs were queried against the Rfam database and matches to known sRNAs are indicated.

Concluding remarks

In this study we have used high throughput molecular technologies and bioinformatic analysis to explore the effect of antibiotics on the global transcriptome of *P. putida* DOT-T1E. The DOT-T1E strain is a non-pathogenic strain that exhibits resistance to high concentration of many antibiotics. This resistance is due to the use of different mechanisms of exclusion such as efflux pumps that remove antibiotics, alterations of membranes that impermeabilize cells, and metabolic breakdown or inactivation of the antibiotics. We explored the effect of exposure to a wide range of antimicrobials and demonstrated very clear antimicrobial-responses at the mRNA and sRNA level. The mRNA expression patterns are in concordance with the described mechanisms of action of the antimicrobials used. For each antibiotic, numerous genes that encode hypothetical proteins were also induced indicating that our knowledge of antibiotic resistance mechanisms is still incomplete.

Recently, a large number of sRNAs have been discovered in bacteria. So far only 16 sRNAs were known to be expressed in *P. putida* DOT-T1E. In this study we have detected 138 novel transcripts encoded in intergenic regions which most likely represent sRNAs. A correlation between the expression profiles of mRNA and sRNA was found, suggesting that some of these sRNAs are likely involved in regulation of antibiotic resistance. These findings open new possibilities in the fight against multiresistant bacteria via the use of sRNAs as antimicrobial targets. We also show the modes of action of eight different antibiotics and the antimicrobial resistance mechanisms of *P. putida* DOT-T1E, these findings may help in the understanding of how antibiotics act against bacteria and how bacteria defend against these external stresses.

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Supplementary material

Table S1. Shared genes between ampicillin, chloramphenicol and kanamycin conditions. Available on: http://1drv.ms/1w7MQCF

Table S2. Shared genes between ciprofloxacin, tetracycline, spectinomycin, gentamicin and rifampicin conditions. Available on: http://1drv.ms/1w7Ndgr

Table S3. mRNAs overexpressed in the presence of chloramphenicol compared with control LB ($P<0.02$).Available on: http://1drv.ms/1w7Nkss

Table S4. mRNAs overexpressed in the presence of kanamycin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NqAu

Table S5. mRNAs overexpressed in the presence of tetracycline compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7Ntfo

Table S6. mRNAs overexpressed in the presence of gentamicin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NzUs

Table S7. mRNAs overexpressed in the presence of spectinomycin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NDDB

Table S8. mRNAs overexpressed in the presence of ciprofloxacin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NMXL

Table S9. mRNAs overexpressed in the presence of rifampicin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NP5U

Table S10. mRNAs overexpressed in the presence of ampicillin compared with control LB ($P<0.02$). Available on: http://1drv.ms/1w7NU9L

Table S11. Oligonucleotides used in this study. Available on: http://1drv.ms/1w7NYGo

Chapter 4

Efflux pump deficient mutants as a platform to search for microbes that produce antibiotics

Molina-Santiago C., Daddaoua A., Udaondo Z., Roca A., Martín J., Pérez-Victoria I., Reyes F. and Ramos J.L. (2014). Efflux pump deficient mutants as a platform to search for microbes that produce antibiotics. To be submmited to Environmental Microbiology.

Summary

Pseudomonas putida DOT-T1E-18 is a strain deficient in the major antibiotic efflux pump and exhibits an overall increased susceptibility to a wide range of drugs as compared to the wild-type strain. We used this strain as a platform to search for microbes able to produce antibiotics that inhibit its growth. A collection of 2400 isolates from soil, sediments and water was generated and a drop assay developed to identify, via growth inhibition halos, strains that prevent DOT-T1E-18 growth on solid LB plates. Thirty five different isolates that produced known and unknown antibiotics were identified. The most potent inhibitor of DOT-T1E-18 growth was an isolated named 250J that, through multilocus sequence analysis, was identified as a *Pseudomonas* sp. strain. Culture supernatants of 250J contain four different xantholysins that prevented growth of Gram-positive bacteria, Gram-negatives and fungi. Two of the xantholysins were produced in higher concentrations and purified. Xantholysin A was effective against *Bacillus, Lysinibacillus* and *Rhodococcus* strains and the effect against these microbes was enhanced when used in combination with other antibiotics such as ampicillin, gentamicin, and kanamycin. Xantholysin C was also efficient against Gram-positive bacteria and showed antimicrobial effect against *Pseudomonas* strains, being its inhibitory effect synergistic with ampicillin, chloramphenicol and gentamicin. We discuss in this work that DOT-T1E-18 is an efficient reporter strain to be used as a platform to identify antibiotic producing strains with high potential against bacteria and fungi.

Introduction

With the increasing antibiotic resistance of microorganisms and the emergence of multidrug-resistant bacteria, a critical situation is arising for immunosuppressed patients and elderly people that need to be treated with effective therapies (Terán *et al.*, 2006; Donadio *et al.*, 2009). Several general kinds of antibiotic resistance have been described in bacteria; including acquired resistance linked to mutations in chromosomal genes or the acquisition of new genes by horizontal transfer (D'Costa *et al.*, 2011; Bernal *et al.*, 2013); adaptive resistance, which involves a temporary increase in the ability of bacterial cells to survive the action of an antibiotic, mainly as the result of alterations in gene and/or protein expression triggered by environmental conditions (Poole, 2012); and intrinsic resistance, which is not related with antibiotic selection but to the specific characteristics of the bacteria, due to the presence of outer membrane lipopolysaccharides that prevent antibiotic entry and the presence of efflux pumps that extrude antibiotics out of the cells (Bernal *et al.*, 2013).

Current challenges in antibiotic therapy are focused in the identification and development of novel and more effective antibiotics to combat multidrug resistant pathogens, particularly those resistant to last-line antibiotic agents (Gwynn *et al.*, 2010). The main classes of antibiotic drugs commonly used today were discovered in the second half of last century and their targets are proteins involved in cell-wall biosynthesis (β-lactams, glycopeptides), cell membrane structures (daptomycin, colistin), type II topoisomerases (fluoroquinolones), ribosome functions (macrolides, aminoglycosides, tetracyclines), transcriptional machinery (rifamycins), and folate biosynthesis (sulfonamides and trimethoprim) (Fernandes, 2006; Lange *et al.*, 2007). Therefore, new antimicrobial agents with a greater potential to address the deficiencies of existing classes of antibiotics are needed to combat bacterial resistance (Gwynn *et al.*, 2010; Laverty *et al.*, 2010). In addition to new antimicrobials discovery, combination therapy with two or more antibiotics is used to prevent or delay the emergence of resistant strains and to take advantage of antibiotic synergism as a potential approach to treat infectious diseases (Zhao *et al.*, 2002). Among the strategies to discover new antimicrobial compounds, chemical modification of existing drugs either with the aim to increase their spectrum of activity or to enhance their activity, and screening methods to identify new natural compounds (e.g., known or unknown target directed, high-

throughput platforms) are often used (Moellering, 2011). One class of compounds that, during the last decades, have attracted an increasing amount of attention due to their promising role as therapeutics or drug leads are short antimicrobial peptides (AMPs), that usually exhibit rapid and efficient antimicrobial toxicity against a wide range of pathogens (Ganz and Lehrer, 1999; Hadley and Hancock, 2010; Hughes and Fenical, 2010; Laverty *et al.*, 2010).

Antimicrobial lipopeptides are bacterial compounds synthesized via non-ribosomal biosynthetic pathways and comprise a peptidyl portion conjugated to a fatty acid forming an acylated peptide (Jerala, 2007). These compounds usually present a cationic or anionic peptide motif which is relevant for their spectra of activity, while fatty acids, alcohols, fatty amines and glyceryl esters vary the degree of antimicrobial activity. It has been shown that acylation of peptide scaffolds improves antimicrobial activity (Shalev *et al.*, 2006; Jerala, 2007). While many Gram-positive bacteria, including different species of the genus *Bacillus*, are reported to produce diverse antimicrobial lipopeptides with different applications in industry (Rodrigues *et al.*, 2006), only few lipopeptides, classified in different groups (e.g., viscosin, amphisin, syringomycin, syringopeptin, or tolaasin), have been reported to be produced by Gram-negative bacteria like *Pseudomonas* (Washio *et al.*, 2011; Mandal *et al.*, 2013) showing activity against Gram-positive bacteria but rarely against Gram-negative ones.

Pseudomonas putida are ubiquitous microorganisms that can be found in soils associated with plant roots and in aquatic ecosystems either in suspension or in biofilms on biotic and abiotic surfaces (Yousef-Coronado *et al.*, 2008; Rodríguez-Herva *et al.*, 2010; Dogan *et al.*, 2011; Jakovleva *et al.*, 2012). *Pseudomonas putida* strain DOT-T1E was isolated from a wastewater treatment plant in Granada and is able to thrive in the presence of high concentrations of organic solvents (Ramos *et al.*, 1995) and toxic compounds such as dyes, heavy metals and a broad array of antimicrobial compounds (Ramos *et al.*, 1998; Terán *et al.*, 2003; Fernández *et al.*, 2012). The main mechanism of resistance towards antibiotics in this strain is their extrusion from the cells to the outer medium through the action of multidrug efflux pumps of the resistance-nodulation-cell division family (RND) (Rojas *et al.*, 2001; Molina-Santiago *et al.*, 2014a). Two of these RND efflux pumps (TtgABC and TtgGHI) have been shown to be involved in antibiotic extrusion, being TtgABC the most relevant with regards to the removal of this kind of compounds in DOT-T1E.

This study describes a new strategy for the discovery of antimicrobial compounds using a *P. putida* DOT-T1E strain deficient in the TtgABC efflux pump, what allowed us to identify highly active antimicrobial compounds. As an example of the effectiveness of the described screening based on TtgABC mutant, we identified *Pseudomonas* sp. strain 250J that is able to produce compounds of the xantholysin family with antimicrobial and antifungal activity.

Materials and methods

Strains and general growth conditions

The bacterial strains used in this study are shown in Table 1. Bacterial cells were grown in liquid LB medium at 30 ºC with shaking in 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^{-1} kanamycin and 20 μg ml^{-1} rifampicin. The concentration of other antibiotics used in this study is indicated in the text. For production of xantholysins, KIDO medium (glycerol 2% [v/v]; sodium L-glutamate monohydrate 1% [w/v]; yeast extract 0.25% [w/v] adjusted to pH 7) was used and cultures were grown at 18 ºC for 3 days (Pascual *et al.*, 2014).

Sample collection

Samples were collected from soils, wastewater, river waters, and river sediments. One loam soil sample was taken at olive fields (Jaén, Spain) in September 2010 [37º 46' 57.93" N, -3º 48' 8.95" W] 574 m. A second soil sample was collected from the garden of Estación Experimental del Zaidín (Granada, Spain) and it was taken in November 2010, [+37°9'56.50"N, −3°35'31.13"O] 678 m. In both cases, we collected 300-500 g of soil from the top 20 cm directly with sterile 50 ml tubes and kept at -20 ºC until they were used.

A water sample was taken from a wastewater treatment plant (Cartagena, Spain) in November 2010 [+37°36'00"N, -0°59'00"O]. This sample was also chosen to search for microbes exposed to a wide range of chemicals derived from domestic disposal and a number of emerging pollutants (including pharmaceuticals and household cleaning products, etc.). Five hundred milliliters of this water were taken and kept at 4 ºC until it was used.

Yellow-red clay samples along the Tinto River (Huelva, Spain) were taken in September 2012 [+37º 18' 41.79'' N, -6º 49' 20.62'' W] 0 m. Tinto River presents extreme conditions [pH 3-4, heavy metal concentrations of 20 μg ml^{-1} Zn, 180 μg ml^{-1} Fe, 19 μg ml^{-1} Cu or 355 μg ml^{-1} S (García-Moyano *et al.*, 2012) and high salt concentration due to the proximity to Atlantic Ocean] and tidal influence.

Sandy samples were also taken along the Odiel River (Huelva, Spain) in September 2012 from [+37º 15' 2.74'' N, -6º 57' 27.28'' W] 0 m to [+37º 12' 58.99'' N, -6º 56' 46.81'' W] 0 m. The estuary of Odiel River (Huelva, Spain) is characterized by tidal influence and high salt concentrations due to their proximity to the mouth of the river to Atlantic Ocean. Water samples (300 ml) were taken from the river channel and kept at 4 ºC until were used. For sediments, we sampled when the tidal influence in the river was maximal and minimal. In each case, composed samples were prepared by taking 100 g sediments in each location with separation between sample points of at least 10 m.

Table 1. Strains used in this study.

Strain	Relevant characteristics	Source of reference
Pseudomonas strains		
P. putida DOT-T1E	Rif^r	Ramos *et al.*, 1998
P. putida DOT-T1E-18	Rif^r, Km^r, *ttgB*::'*phoA*-Km	Ramos *et al.*, 1998
P. putida DOT-T1E-PS28	Rif^r, Sm^r, *ttgH*ΩSm	Rojas *et al.,* 2001
P. aeruginosa PAO1	Wild type, prototroph, Ap^r	Stover *et al.,* 2000
Pseudomonas sp. strain 250J	Xantholysin producer isolated from the garden of Estación Experimental del Zaidín (Granada, Spain)	This study
P. mendocina	Isolated from olive soil (Jaén, Spain)	This study
P. nitroreducens	Isolated from Tinto River (Huelva, Spain)	This study
Bacillus strains		
Bacillus sp.	Isolated from Tinto River (Huelva, Spain)	This study
B. subtilis	Isolated from Muelle del Tinto (Huelva, Spain)	This study
Other strains		
Lysinibacillus fusiformis	Isolated from Muelle del Tinto	This study

	(Huelva, Spain)	
Rhodococcus erytrophyla	Isolated from Tinto River (Huelva, Spain)	This study
E. coli K12	Wild-type	Kar *et al.,* 2005

Isolation media

The following isolation media with an adjusted pH 7.0 – 7.5 were used: M9 minimal medium with different carbon sources (syringic acid, vanillic acid, *o*-anisic acid, *p*-anisic acid, ferulic acid, veratric acid, *p*-cumaric acid, 2-hydroxyphenylacetic acid, 2,4-dihydroxybenzoic acid, phloroglucinol, benzoic acid) (Abril *et al.*, 1989); IM1 (humic acid agar with sea water): humic acid (1 g), K_2HPO_4 (0.5 g), $FeSO_4$ x $7H_2O$ (1 mg), agar (20 g), vitamin B solution (1 ml), natural sea water (0.5 l) and distilled water (0.5 l) (Zotchev, 2012); IM2: glycerol (0.5 g), starch (0.5 g), sodium propionate (0.5 g), KNO_3 (0.1 g), asparagine (0.1 g), casein (0.3 g), K_2HPO_4 (0.5 g), $FeSO_4$ x $7H_2O$ (1 mg), agar (20 g), vitamin B solution (1 ml), natural sea water (0.5 l) and distilled water (0.5 l) (Zotchev, 2012); IM3 (chitin agar with sea water): chitin (Sigma), K_2HPO_4 (0.5 g), $FeSO_4$ x $7H_2O$ (1 mg), agar (20 g), vitamin B solution (1 ml), natural sea water (0.7 l) and distilled water (0.3 l) (Bredholt *et al.*, 2008); IM4: malt extract (1 g), glycerol (1 g), glucose (1 g), peptone (1 g), yeast extract (1 g), agar (20 g), natural sea water (0.5 l) and distilled water (0.5 l) (Bredholt *et al.*, 2008); IM5-soil agar: filtered soil extract (1 l), agar (20 g), pH 7.0 (soil extract was prepared by mixing 200 g soil in 1 l water, followed by boiling the mixture for 30 min); 1 ml of vitamin complex solution [calcium pantothenate (10 mg), nicotinic acid (10 mg), thiamin chloride (1 mg), biotin (1 mg), tap water (20 ml)] was added into soil agar; IM6: IM5-soil agar with 3% (w/v) sea salt added; IM7: modified organic agar 2 Gause: peptone (5 g), tryptone (3 g), glucose, (10 g), NaCl (5 g), tap water (1 l) (Engelhardt *et al.*, 2010), IM8: modified organic agar 2 Gause with 3% (w/v) sea salt added; IM9-mineral agar 1 Gause: starch-soluble (20 g), K_2HPO_4 (0.5 g), $MgSO_4$ (0.5 g), KNO_3 (1.0 g), NaCl, (0.5 g); FeSO4 x $7H_2O$ (0.01 g), tap water (1 l); IM10: modified organic agar 2 Gause supplemented with tobramycin (10 μg ml^{-1}) (Terekhova *et al.*, 1991); IM11-½ ISP2: malt extract (5 g), yeast extract (2 g), glucose (2 g), natural sea water (0.5 l) and distilled water (0.5 L) (Hakvag *et al.*, 2008); IM12-Kusters streptomycete isolation medium (modified); glycerol (10 g), casein (0.3 g), KNO_3 (2 g), $FeSO_4$ x

7H_2O (0.25 mg), H_2SO_4 (0.5 mg), natural sea water (0.5 l) and distilled water (0.5 l); IM13-medium A (9K mineral medium supplemented with 1% (w/v) glucose, and 1% (w/v) yeast extract); IM14-medium I (9K medium supplemented with 0.1% (w/v) bactotryptone, 1% (w/v) malt extract, 1% (w/v) glucose, 0.5% (w/v) yeast extract, and 0.5% (w/v) sucrose) (Silverman and Lundgren, 1959); IM15-medium J (9K medium supplemented with 0.1% (w/v) casamino acids, 0.1% (w/v) bactopeptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) sucrose); IM16-medium F (1 mM KH_2PO_4, 1 mM $MgCl_2$, 1.5 mM $(NH_4)_2SO_4$, 0.5% (w/v) glucose, 0.05% (w/v) malt extract, 0.5% (v/v) trace metals) (Silverman and Lundgren, 1959).

Inoculated media were incubated at 30 ºC for 24 h, 48 h, 72 h, 1 week and 2 weeks with agitation (200 rpm) and at the indicated times aliquots were collected and plated in the same isolation medium that the one used for enrichment but supplemented with agar (2% w/v). Different colonies were selected according to their apparent phenotype, such as color, roughness, size and morphology in order to keep as many different bacteria as possible creating a collection of around 2400 microorganisms.

Phylogenetic analysis

Genomic DNA was used as a template for polymerase chain reaction (PCR) amplification. For 16S ribosomal RNA (rRNA) gene amplification, primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') were used (Lane, 1991). PCR amplifications were carried out under the following conditions: initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 30 sec, 46 ºC for 30 sec, and 72 ºC for 2 min, with a final extension at 72 ºC for 7 min. In all cases, the reaction mixture (50 μl) contained deoxyribonucleotide triphosphate (0.2 mM each), 1 x reaction buffer (20 mM Tris pH 8.4, KCl 50 mM), $MgCl_2$ (1.7 mM), primers (0.1 μM each), and Taq DNA polymerase (1.25 units). PCR products were analyzed by agarose gel electrophoresis, which were purified using Qiaquick gel extraction kit (Qiagen) and were sequenced directly using primers 27f and 1492r. The partial 16S rRNA gene sequences were analyzed using BLAST (Altschul *et al.*, 1997) and named according to their closest neighbors.

Antimicrobial producer identification

Pseudomonas putida DOT-T1E-18 (TtgABC mutant) reporter strain was routinely grown aerobically at 30 ºC. Antimicrobial activity of the isolated bacteria against the reporter strain was assessed with a colony overlay assay. The indicator bacteria was spread on LB plates, allowed to dry and after that, isolated bacterial colonies from an overnight pre-culture were loaded. Plates were incubated for 24 h and inhibition zones around the spots were scored as positive. In the case of a positive result, liquid antimicrobial activity screening assays were done in 96-well plates. For that, in the wells of a microdilution plate, 65 µl of filtrated supernatant was mixed with 25 µl LB and 10 µl of the reporter bacterium was added to reach an initial OD_{660nm} of 0.05. These plates were incubated at 30 ºC with agitation (200 rpm) for 24 h and growth was determined. Inhibition was scored as positive if the turbidity was 10-fold lower than in the control without filtrate supernatant. Experiments were repeated at least 3 times.

Checkerboard analysis

Standard powder forms of ampicillin, chloramphenicol, gentamicin and kanamycin were stored at 2 to 8 ºC until use. The stock solutions and serial two-fold dilutions of each drug to at least double the MIC were prepared according to the recommendations of NCCLS immediately prior to testing. A total of 100 µl of LB broth inoculated with the bacterial inoculum at a 0.5 McFarland turbidity was distributed into each well of the microdilution plates. The first drug of the combination was serially diluted along the ordinate, while the second drug was diluted along the abscissa and the plates were incubated at 30 ºC or 37 ºC for 24 h at 200 rpm.

According to the NCCLS guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected by reading turbidity at 600 nm. Synergy is more likely to be expressed according to ∑FICs that were calculated as follows: ∑FIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ∑FIC is ≤ 0.5, additive when the ∑FIC is > 0.5 to ≤ 1, no interaction (indifference) when ∑FIC is > 1 to ≤ 4 and antagonistic when the ∑FIC is ≥ 4 (Sopirala *et al.*, 2010).

Minimum inhibitory concentration (MIC)

MIC assays of DOT-T1E, T1E-18 and T1E-PS-28 were performed in liquid LB medium 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^{-1}); chloramphenicol (3,000 μg ml^{-1}); norfloxacin (200 μg ml^{-1}); erythromycin (3,000 μg ml^{-1}); gentamicin (1,000 μg ml^{-1}), kanamycin (2,500 μg ml^{-1}) and ampicillin (10,000 μg ml^{-1}). In the cases of xantholysin A and C, a concentration of 2,000 μg ml^{-1} was used. 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 were spread with a suspension of approximately 10^8 CFU/ml of wild-type *P. putida* DOT-T1E or DOT-T1E-18 mutant strain 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.

Extraction and purification of Xantholysin A and Xantholysin C

A culture of *Pseudomonas* sp. strain 250J (1 l) grown in 2 L flasks for three days at 18 ºC was extracted acidifying the fermentation broth with HCl to a pH of 2-3. After that, methyl ethyl ketone (EtCOMe) was added and three steps of 10 min centrifugation were done. After each centrifugation, the organic phase was transferred to glass tubes. The sample was concentrated in an orbital shaker and resuspended in DMSO. The solution was loaded onto a semi-preparative HPLC column (Zorbax SB-C8, 21.2_250 mm, gradient H_2O 0.1% (v/v) CF_3COOH (TFA)/MeCN 0.1% (v/v) TFA from 5 to 100% (v/v) of MeCN/TFA in 45 min, 3.6 ml min^{-1}, UV detection). The peak eluting at 31 min was repurified on the same column with a 68% isocratic MeCN/TFA yielding 16 mg of xantholysin A and 5 mg of xantholysin C.

Preparation and analysis of Marfey derivatives

Xantholysin A was hydrolyzed by heating in HCl (6 N, 300 µL) at 110 ºC for 16 h (total hydrolysis) and in HCl (0.5 N) at 110 ºC for 7 h (partial hydrolysis). In the case of partial hydrolysis, fragments of interest were purified by HPLC using a gradient of 30-80% (v/v) of acetonitrile in water. After cooling, the solution was evaporated to dryness and re-dissolved in H_2O (50 µL). To the peptide acid hydrolysate solution (or to 50 µL of a 50 mM solution of the respective amino acid standard) were added $NaHCO_3$ (1 M; 20 µL) and then a 1% (w/v) solution (100 µL) of FDVA (Nα-(2,4-dinitro-5-fluorophenyl)-L-valinamide, a variant of Marfey's reagent) in acetone. The mixture was incubated for 1 h at 40 ºC. The reaction was stopped by addition of HCL (1 N, 20 µL). A 3 µL aliquot of these solutions were analyzed by HPLC-UV-MS (Zorbax SB-C8 column, 2.1 × 30 mm, 5 µm, 40 ºC, 500 µL/min; linear gradient: 0 min 22% B, 33 min 60% B, mobile phase and detection as described above for the general LC-UV-MS analysis). Retention times (min) of the FDVA amino acid derivatives used as standards were as follows: L-Ser (7.6), D-Ser (9.5), L-Glu (8.9), D-Glu (11.6), L-Val (17.1), D-Val (25.1), L-Leu (20.8), D-Leu (29.2), L-Ile (20.8) and D-Ile (29.2).

Retention times (min) of the observed peaks in the HPLC trace of the FDVA-derivatized hydrolysis products of xantholysin A were as follows: D-Ser (9.7), L-Glu (9.4), D-Glu (11.9), D-Val (25.1), L-Leu (20.8), D-Leu (29.1), L-Ile (20.8) and D-Ile (29.1). Due to the close retention times observed for L-Ile, L-Leu, D-Ile and D-Leu, the presence of L- or D-Ile was confirmed using a second HPLC method with isocratic elution (33% B during 60 min). Under these conditions, retention times of 19.1 and 19.9 min were obtained for L-Ile and L-Leu, respectively, and 50.2 and 51.5 were obtained for D-Ile and D-Leu, respectively.

Results and discussion

Method for the identification of antimicrobial producer bacteria using *P. putida* DOT-T1E-18 as a reporter

In *P. putida* strain DOT-T1E, extrusion of antimicrobial compounds is mainly achieved through the action of RND efflux pumps (Mosqueda and Ramos, 2000; Rojas *et al.*, 2001; Godoy *et al.*, 2010; Fernández *et al.*, 2012). TtgABC is the main efflux pump responsible of antibiotic extrusion in this strain, with TtgGHI playing

a secondary role although, through its synergistic action, helps to confer high antibiotic resistance to DOT-T1E (Molina-Santiago *et al.*, 2014b). MIC concentrations and inhibition zones for different antibiotics of the wild-type and mutant strains (DOT-T1E-18, DOT-T1E-PS28) were determined. We found that for the complete set of antibiotics used, the minimal concentration necessary to inhibit T1E-18 growth was significantly lower than for the wild-type strain and the DOT-T1E-PS28 strain mutated in TtgGHI efflux pump (Table 2). Similar results were found when inhibition zone assays were done, as the halos in response to a given concentration of an antibiotic were largest in the case of the DOT-T1E-18 mutant strain (Figure S1). We reasoned that the fact of T1E-18 being more sensitive to antibiotics than the parental strain could be exploited to identify production of antimicrobials by the collection of 2400 environmental isolates described in Materials and Methods.

Table 2. Minimum inhibitory concentrations (μg ml^{-1}) of antibiotics (tetracycline, nalidixic acid, ampicillin, chloramphenicol, gentamicin, piperacillin, amikacin, ceftriaxone and ticarcillin) for *P. putida* DOT-T1E, DOT-T1E-18 and DOT-T1E-PS28 strains at 30 ºC.

Antibiotic (μg ml^{-1})	DOT-T1E	DOT-T1E-18	DOT-T1E-PS28
Tetracycline	8	<1	8
Nalidixic acid	250	7.8	250
Ampicillin	625	200	625
Chloramphenicol	300	70	300
Gentamicin	4	1	2
Piperacillin	35	17	35
Amikacin	2	<1	2
Ceftriaxone	11	4	11
Ticarcillin	187	4	187

We performed inhibition zone assays to determine the antimicrobial activity of the environmental isolates against DOT-T1E-18. In these assays, we spread the indicator strain on LB plates, and once dried, we dropped 3 μl of each isolated bacteria on the dried surface of the LB plates. After 24 h incubation at 30 ºC,

plates were inspected for the appearance of inhibition halos. We found that 150 bacteria of the collection produced an inhibition halo, and they were considered as positive antimicrobial producers (Figure S2). To further test the production of antimicrobials, culture supernatants were harvested and tested to confirm inhibition growth in 96 well-plates. For these assays, overnight cultures were centrifuged and filtrated and 65 μl of supernatants were placed in wells with 25 μl of LB and 10 μl of the diluted DOT-T1E-18 reporter strain. After 24 h, growth was measured to determine the antimicrobial effect of the supernatants to confirm the activity. In most of the cases, culture supernatants conserved the inhibitory potential observed with the drop of cells assayed.

Phylogenetic analysis

To learn about the potential phylogenetic adscription of the above 150 isolates, fragments of the 16S rRNA gene were PCR-amplified and sequenced. BLAST analysis revealed that some of the isolates could be sibling since they shared 100% of the sequences. This screening reduced the number of potential different microbes to 35 different strains. Of these strains, *Bacillus* and *Pseudomonas* species were the most represented with 12 and 8 different members respectively, and bacteria of the genus *Enterobacter*, *Raoultella*, *Alishewanella, Rhodococcus*, *Aeromonas*, *Vibrio*, *Shewanella, Acinetobacter, Alcaligenes, Cupravidus and Lysinibacillus* were also identified. We also found 8 strains whose 16S rRNAs matched sequences of strains deposited in the databases as "uncultured" microbes (Table S1).

Antimicrobial compound detection

Extracts of fermentation broths of the 35 selected strains were analyzed by LR-HPLC-MS to detect potential new antimicrobial compounds. From all the strains studied, we found that a number of strains produced antibiotics already described such as, i.e. *Alishewanella* sp. strain 255W, which produced compounds of the valin-surfactins and plipastatin families (Figure S3 and Table S2). We also identified some strains producing unknown antimicrobial compounds or compounds described in the literature as rarely produced by bacteria. For instance, we identified that *Lysinibacillus fusiformis* strain 249MT produced erythrolic acid D and molecules with a known molecular formula which have been described to be produced by fungi, plants and even animals, but no bacteria (Figure S4 and Table S3). We found that *Vibrio* sp. strain 225TR produced two unknown compounds

together with the already discovered serratomolide C and 4-deoxy thiomarinol H (Figure S5 and Table S4). Finally, we identified *Pseudomonas* sp. strain 250J that produced a whole new family of four uncharacterized compounds at the time we initiated this study; of these chemicals the majoritarian one was xantholysin A (Figure 1 and Table S5).

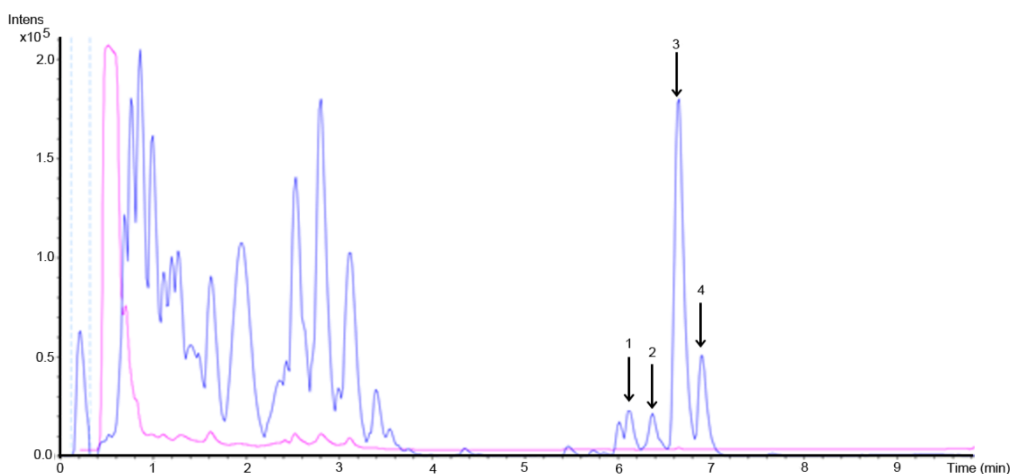

Figure 1. HPLC-MS profile of *Pseudomonas* sp. 250J culture extracts. Numbers 1 to 4 indicate the four xantholysins produced by *Pseudomonas* sp. strain 250J.

Isolation, purification and absolute configuration of xantholysins

The producing bacterium, *Pseudomonas* sp. strain 250J, was fermented for 3 days in KIDO medium. One liter fermentation was extracted with MEK and this extract was purified by repeated semi-preparative HPLC. Four compounds of the xantholysin family were detected (Figure 1): 1775.08 Da (xantholysin A), 1761.07 Da (xantholysin B), 1802.0 Da (xantholysin C), and 1775.09 Da (xantholysin D). Xantholysin A ($C_{84}H_{146}N_{18}O_{23}$) and xantholysin C ($C_{86}H_{148}N_{18}O_{23}$) were successfully isolated in sufficient quantity (16 mg and 5 mg respectively) to allow adequate NMR and MS-MS analysis (Figure S6 and S7). The results confirmed that these compounds were xantholysin A and xantholysin C (Li *et al.*, 2013), with the only single difference in the lipid tail moiety consisting of 3-hydroxydodec-5-enoate in xantholysin C rather than 3-hydroxydecanoate in xantholysin A. This difference accounts for the 26 Da mass increase in xantholysin C (Li *et al.*, 2013).

To dig in the configuration of the amino acid residues in xantholysin A we used the Marfey's methodology (Marfey, 1984). Total and partial hydrolysis of the sample with HCl 6 N and 0.5 N, respectively (110 ºC, overnight), and derivatization of the hydrolysates were done with 1-fluoro-2,4-dinitrophenyl-5-L-valinamide (L-FDVA). This reagent reacts stoichiometrically with the primary amides in L- or D-amino acids, resulting in derivatives with different polarity which led us to identify most of the L- and D-amino acids configurations on the basis of comparison of their retention times with those of derivatized standards. Marfey analysis (Figure S8-S11) led us to identify by HPLC-MS that the serine and the two valine residues were D-amino acids. The analysis also permitted us to identify that Ile has an L-Ile configuration. In the case of Glu-Gln and Leu residues, we found relations of L- and D- amino acids of 1-4 and 3-2, respectively. Partial hydrolysis and purification of the fragments identified the position of two D-Gln and one D-Glu and also one L-Leu and one D-Leu (Figure S12-S13), although this analysis was not sufficient to determine the complete structure of xantholysin A.

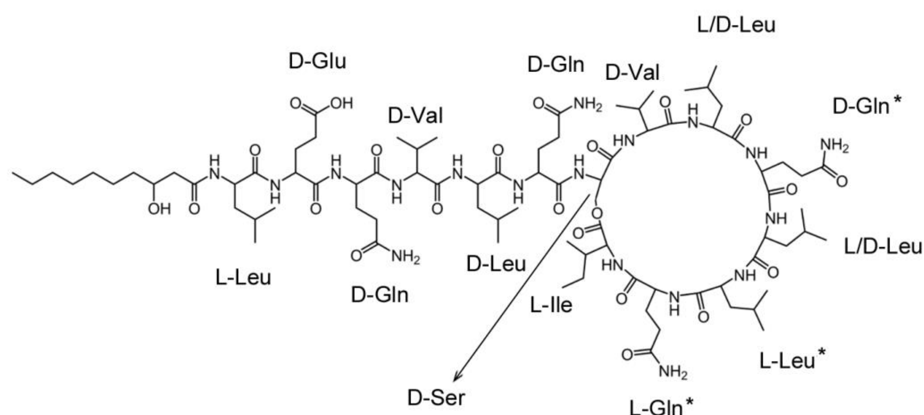

Figure 2. Proposed conformational structure of xantholysin A after Marfey analyses.

In an attempt to resolve the complete structure, bioinformatics analysis performed with NaPDoS (Ziemert *et al.*, 2012) suggested that domains C12 and C13 (belonging to amino acids Leu and Gln) are LCL-C domains, which catalyze peptide bond formation between two L-amino acids. This analysis supported that

Leu and Gln in those positions are likely L-amino acids. The *in silico* analysis revealed not provided data on the potential configuration of the the last Gln residue and of the two Leu residues. Figure 2 shows the potential structure of xantholysin A although the L- or D- configurations of Leu9 and Leu11 are unknown at present.

Antimicrobial activity of xantholysins

To assess the antimicrobial activity of the supernatant of *Pseudomonas* sp. strain 250J containing xantholysins, we evaluated its potential against Gram-negative and Gram-positive bacteria, such as different *Pseudomonas* species, *B. subtilis*, *Rhodococcus erythrophyla*, *E. coli* K12 and *Lysinibacillus fusiformis*. Culture supernatant of 250J strain was used in growth inhibition assays (Table 3).

Table 3. Inhibition growth activity of 250J supernatant and pure xantholysin A and C against a wide range of bacteria.

Strains	250J supernatant growth inhibition (%)	MIC (μg ml^{-1}) Xantholysin A	MIC (μg ml^{-1}) Xantholysin C
P. putida DOT-T1E	79	500	62,5
P. putida DOT-T1E-18	83	500	62,5
E. coli K12	76	>1000	>500
P. aeruginosa PAO 1	68	>1000	62.5-125
P. putida KT2440	75	500	125
Bacillus sp.	95	50	250
Lysinibacillus fusiformis	100	25	31.25
Rhodococcus erythrophyla	100	15.7	7.5
P. mendocina	80	500	125
P. nitroreducens	85	250	125

Purified xantholysin A inhibited growth of Gram-positive bacteria *Bacillus* sp., *Lysinibacillus fusiformis* and *Rhodococcus erythrophyla* (MIC of 15-50 μg ml^{-1}). Xantholysin A also inhibited growth of Gram-negative strains but higher concentrations were needed, i,e., *Pseudomonas* strains (MIC of 250-500 μg ml^{-1}), *E. coli* and *P. aeruginosa* (>1000 μg ml^{-1}) (Table 3). In contrast, when we used purified Xantholysin C we observed growth inhibition effect against all *Pseudomonas* tested

(Table 3) in ranges between 62.5 and 125 μg ml^{-1}) and similar effects than xantholysin A against Gram-positives.

Synergistic effect of xantholysin A and C with other antibiotics

To analyze a hypothetical role of xantholysins as adjuvant of antibiotics against different strains, checkerboard assays were done to study synergism of xantholysins A and C with classical antimicrobial compounds like ampicillin, gentamicin, chloramphenicol and kanamycin (Table 4). According to the results shown in Table 4, xantholysin A presented a synergistic effect (FIC ≤ 0.5) when was supplied with ampicillin (*Bacillus* sp. and *Rhodococcus erythrophyla*), gentamicin (*Bacillus* sp.), chloramphenicol (*P. putida* DOT-T1E) and kanamycin (*Rhodococcus erythrophyla*). As is shown in Table 4, additive effects were also found with xantholysin A in combination with these antibiotics and in other cases synergistic effects were not found. In the case of xantholysin C, we only observed synergism when supplied with chloramphenicol against DOT-T1E, with ampicillin and gentamicin against *Bacillus* sp., and with gentamicin against *E. coli* K12 (Table 4).

Table 4. Synergy of A) xantholysin A and B) xantholysin C with ampicillin, chloramphenicol, kanamycin and gentamicin against Gram-positive and Gram-negative bacteria. Legend: A – additive; S – synergy; I – indifference. Numbers in parenthesis indicate FIC values.

A)

	Ap	Cm	Km	Gm
P. putida DOT-T1E	A (0.98)	S (0.49)	A (0.73)	I
P. putida KT2440	I	I	I	I
Bacillus sp.	S (0.39)	nt	I	S (0.5)
Rhodococcus erythrophyla	S (0.4)	A (0.75)	S (0.45)	I
E. coli K12	A (1)	nt	A (1)	A (1)
P. aeruginosa PAO	I	I	I	S (0.5)

B)

	Ap	**Cm**	**Km**	**Gm**
P. putida DOT-T1E	I	S (0.44)	A (0.96)	I
P. putida KT2440	I	I	A (0.98)	A (0.98)
Bacillus sp.	S (0.48)	nt	A (0.94)	S (0.49)
Rhodococcus erythrophyla	A (0.9)	A (0.9)	I	A (0.9)
E. coli K12	A (1)	nt	A (1)	S (0.5)
P. aeruginosa PAO	I	I	I	I

Concluding remarks

The emerging of multiresistant bacteria and the lack of new antibiotics to combat them has evolved as a serious risk for humanity. In this study, we provide a new platform for the detection of new antimicrobial compounds based on a *P. putida* DOT-T1E strain deficient in the TtgABC efflux pump, the main element related with antibiotic extrusion. The mutant strain is more sensitive to antibiotics, what led us to detect antimicrobials in an easy way.

Out of the 2400 isolated obtained from different environmental niches; we identified 35 strains that belong to different genus, with dominance of *Bacillus* and *Pseudomonas* strains, with antimicrobial activity against DOT-T1E-18 (TtgABC mutant). HPLC-MS analyses of the culture strains led us to identify different known and unknown compounds, thus confirming that the platform fulfills the proof-of-concept for detection of microbes that produce antibiotics.

Of the 35 strains, *Pseudomonas* sp. 250J was able to produce 4 compounds of the xantholysin family. Purified xantholysin A and C were used to elucidate their structures. We also analyzed their antimicrobial activities showing both compounds similar patterns against Gram-positive bacteria and, in the case of xantholysin C, it also exhibit activity against Gram-negative bacteria. Xantholysin C is a new cyclic lipodepsipeptide with activity against *Pseudomonas* species. We showed a synergistic effect of both compounds with classical antibiotics against Gram-positive and Gram-negative strains.

Our results support future studies to determine the potential use of these compounds in clinical assays.

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Supplementary material

Figure S1. Inhibition halos produced by different antibiotics against (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-18, and (C) *P. putida* DOT-T1E-PS28.

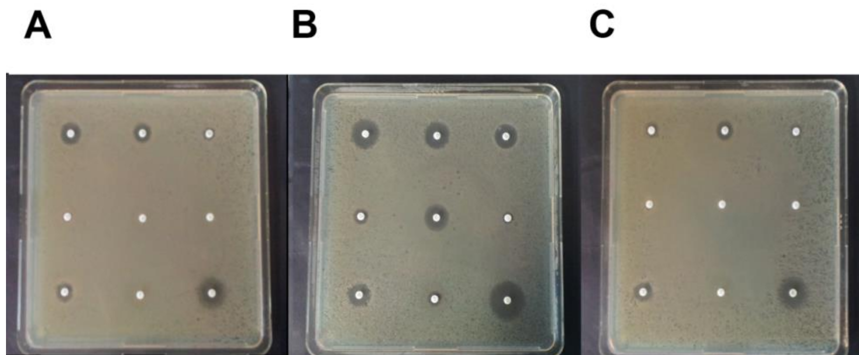

Figure S1. Inhibition halos produced by different antibiotics against (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-18, and (C) *P. putida* DOT-T1E-PS28. From left to right and top to bottom: ofloxacin, ciprofloxacin, amoxicillin, ticarcillin, ampicillin, chloramphenicol, ceftazidime, erythromycin and tetracycline.

Figure S2. Inhibition halos produced by different bacteria against the reporter strain DOT-T1E-18

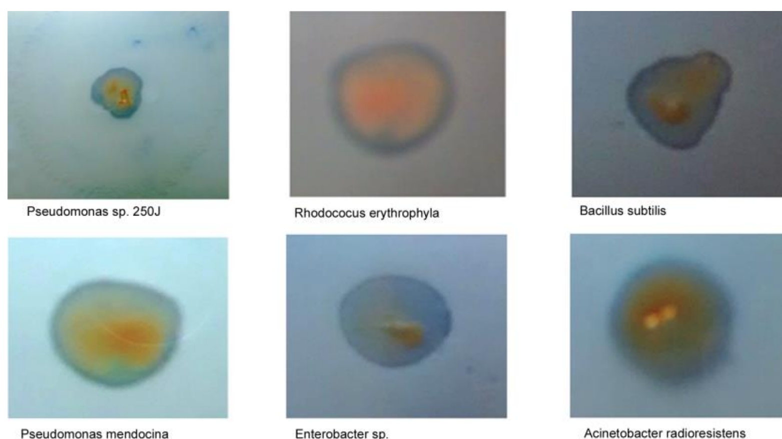

Figure S2. Inhibition halos produced by different bacteria against the reporter strain DOT-T1E-18. From left to right and top to bottom: *Pseudomonas* sp. 250J, *Rhodococcus erythrophyla*, *B. subtilis, P. mendocina, Enterobacter* sp. and *Acinetobacter radioresistens*.

Figure S3. HPLC-MS profile of 255W culture extracts

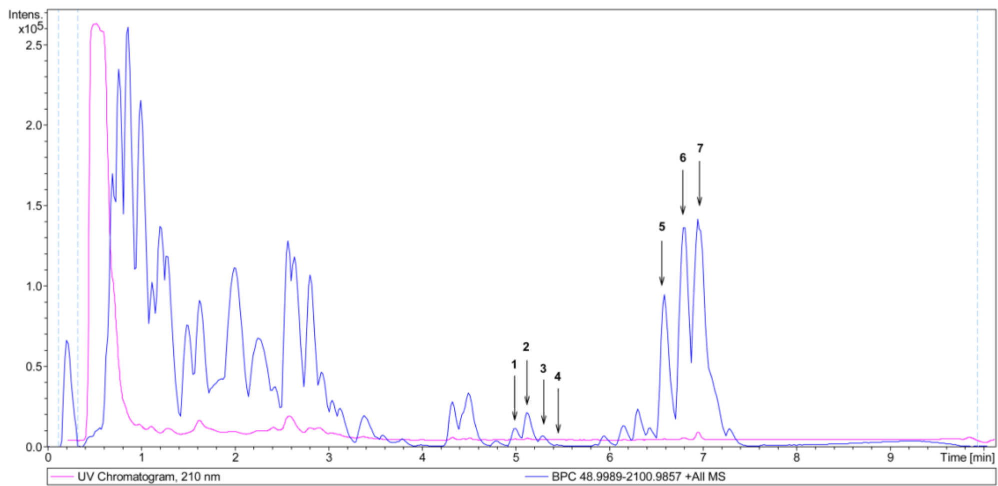

Figure S3. HPLC-MS profile of 255W culture extracts. Numbers 1 to 7 indicate antimicrobial compounds produced by 255W strain.

Figure S4. HPLC-MS profile of 249MT culture extracts

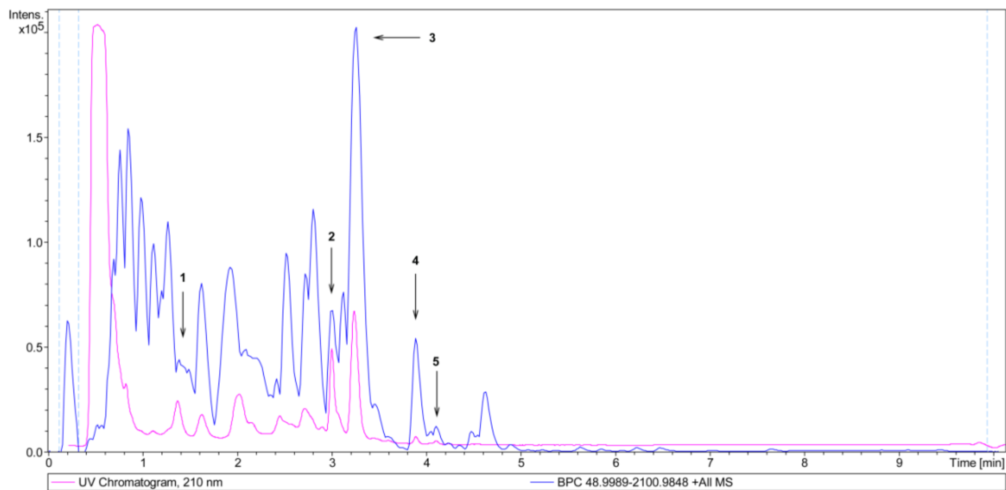

Figure S4. HPLC-MS profile of 249MT culture extracts. Numbers 1 to 5 indicate antimicrobial compounds produced by 249MT strain.

Figure S5. HPLC-MS profile of 225TR culture extracts

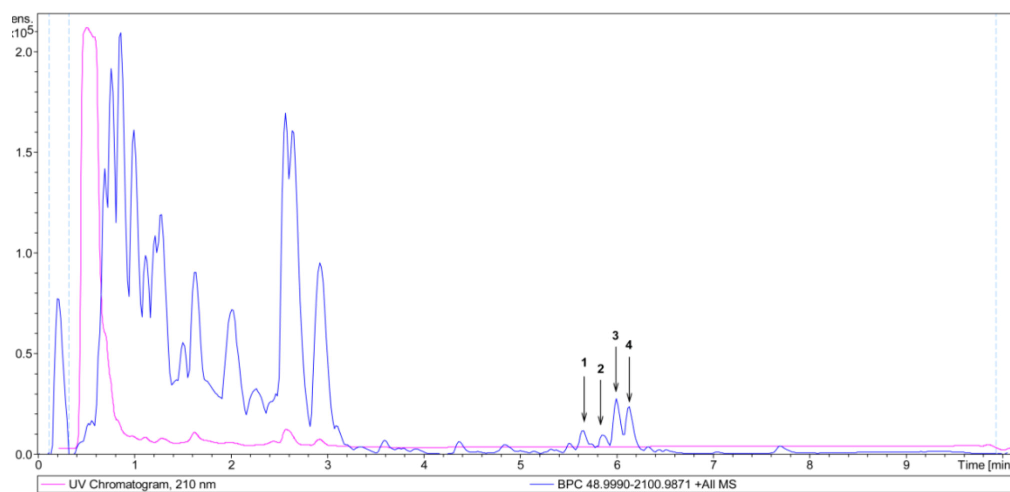

Figure S5. HPLC-MS profile of 225TR culture extracts. Numbers 1 to 4 indicate antimicrobial compounds produced by 225TR strain.

Figure S6. RMN analysis of xantholysin A prepared in $MeOH_d$

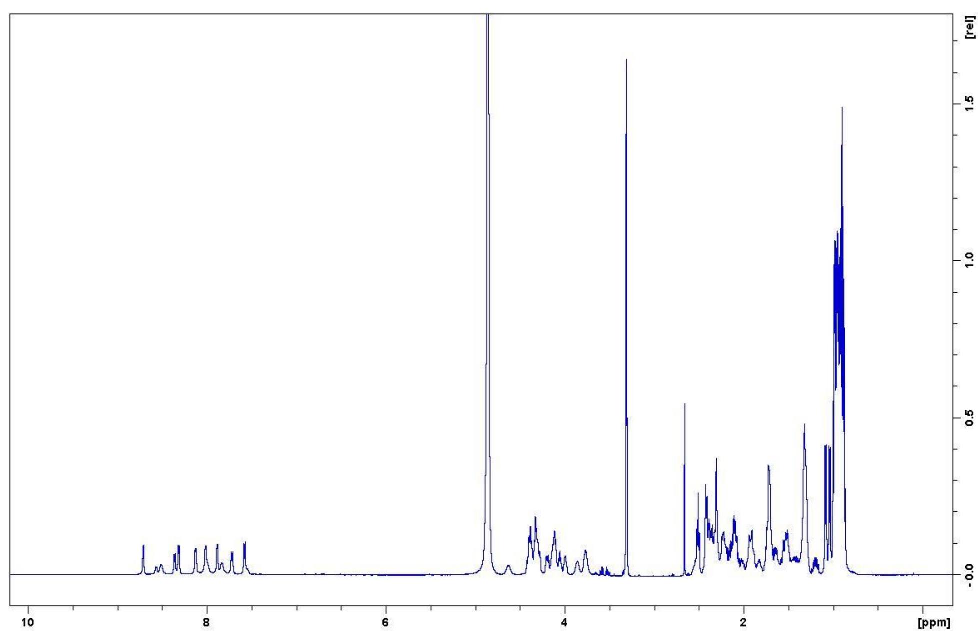

Figure S6. RMN analysis of xantholysin A prepared in $MeOH_d$

Figure S7. MS-MS analysis of xantholysin C

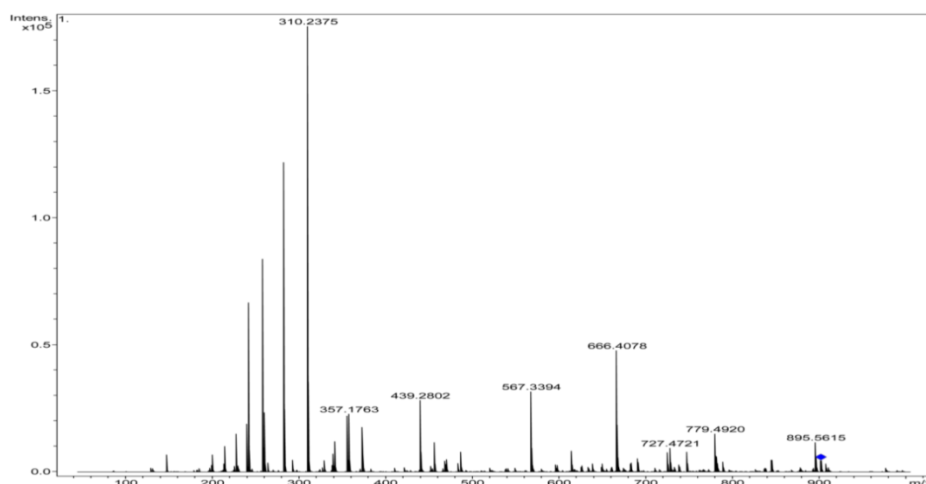

Figure S7. MS-MS analysis of xantholysin C. The analysis were performed to confirm the difference in the lipid tail between xantholysin A and xantholysin C, proposed by Li *et al.*, (2013).

Figure S8. Marfey analysis of D-Ser, (A) xantholysin A MS, (B) positive ion of D-Ser in xantholysin A, and (C) positive ion of D-Ser control.

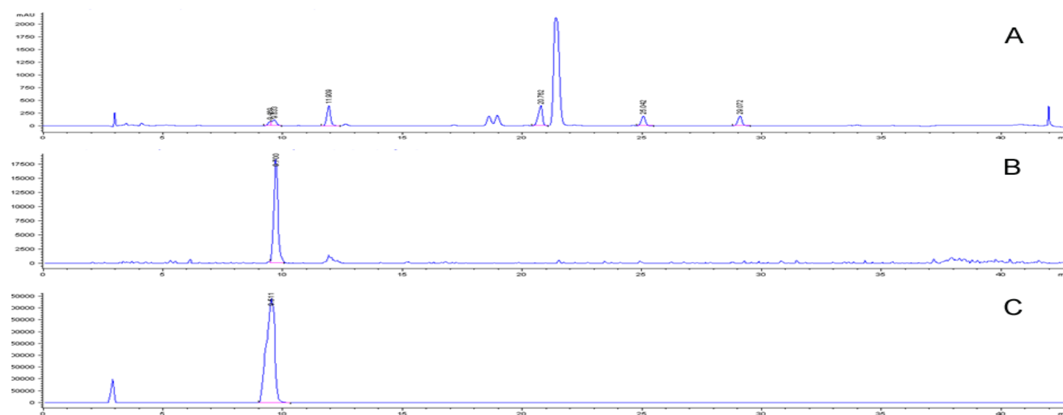

Figure S8. Marfey analysis of D-Ser, (A) xantholysin A MS, (B) positive ion of D-Ser in xantholysin A, and (C) positive ion of D-Ser control.

Figure S9. Marfey analysis of D-Val. (A) xantholysin A MS, (B) positive ion of D-Val in xantholysin A, and (C) positive ion of D-Val control.

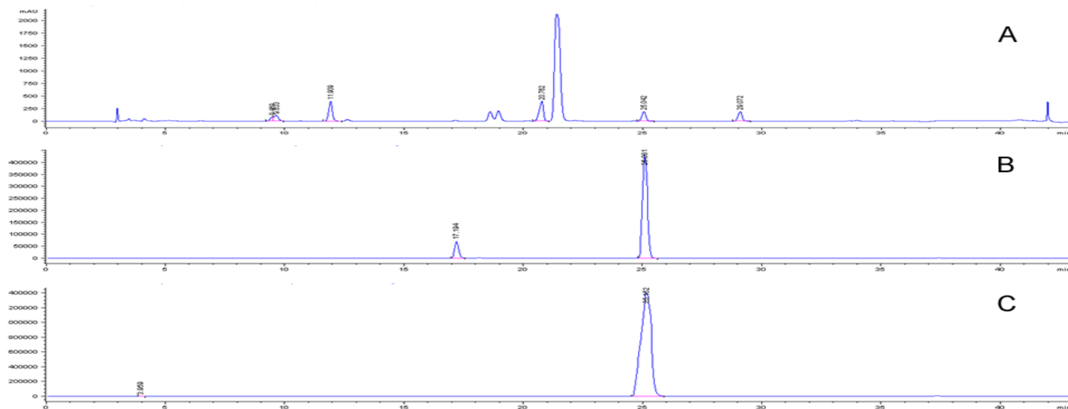

Figure S9. Marfey analysis of D-Val. (A) xantholysin A MS, (B) positive ion of D-Val in xantholysin A, and (C) positive ion of D-Val control.

Figure S10. Marfey analysis of L- and D-Glu/Gln. (A) xantholysin A MS, (B) positive ion of D- and L-Glu/Gln in xantholysin A, (C) positive ion of D-Glu/Gln control, and (D) positive ion of L-Glu/Gln control.

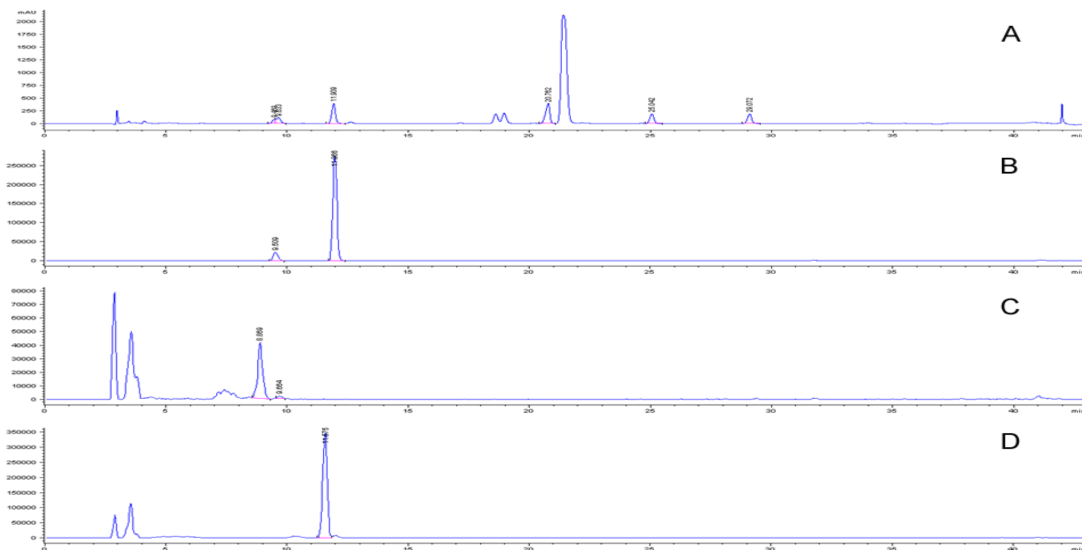

Figure S10. Marfey analysis of L- and D-Glu/Gln. (A) xantholysin A MS, (B) positive ion of D- and L-Glu/Gln in xantholysin A, (C) positive ion of D-Glu/Gln control, and (D) positive ion of L-Glu/Gln control.

Figure S11. Marfey analysis of D- and L-Ile/Leu. (A) xantholysin A MS, (B) positive ion of D- and L-Leu/Ile in xantholysin A, (C) positive ion of D-Leu control, (D) positive ion of L-Leu control, (E) positive ion of D-Ile control, and (F) positive ion of L-Ile control.

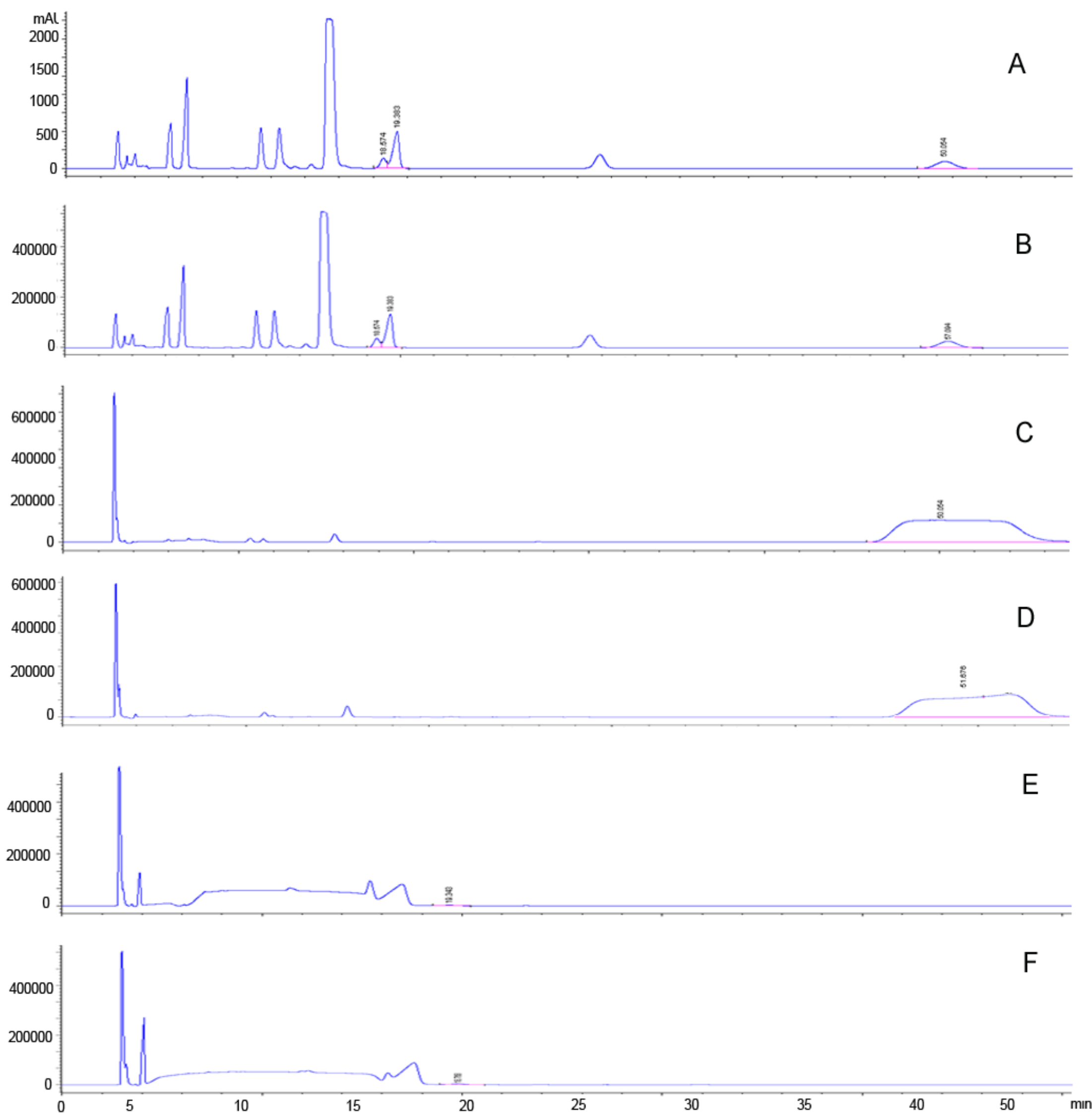

Figure S11. Marfey analysis of D- and L-Ile/Leu. (A) xantholysin A MS, (B) positive ion of D- and L-Leu/Ile in xantholysin A, (C) positive ion of D-Leu control, (D) positive ion of L-Leu control, (E) positive ion of D-Ile control, and (F) positive ion of L-Ile control.

Figure S12. Marfey analysis of a fragment of 301 Da from partial hydrolysis. (A) fragment of 301 Da MS, (B) positive ion of L-Leu in fragment of 301 Da, (C) positive ion of L-Leu control.

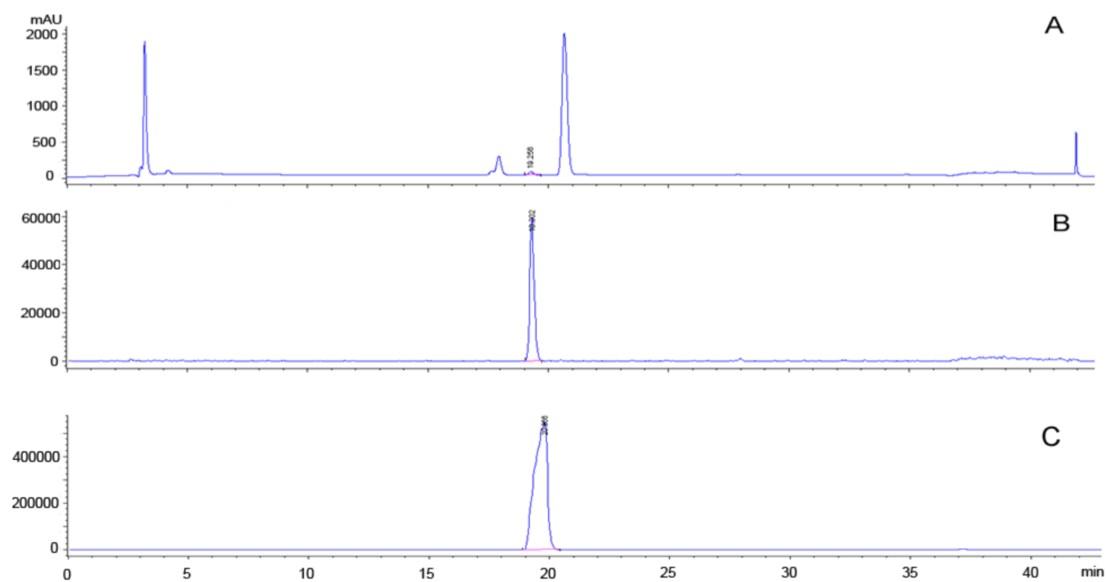

Figure S12. Marfey analysis of a fragment of 301 Da from partial hydrolysis. (A) fragment of 301 Da MS, (B) positive ion of L-Leu in fragment of 301 Da, (C) positive ion of L-Leu control.

Figure S13. Marfey analysis of a fragment of 900 Da from partial hydrolysis. (A) Fragment of 900 Da MS, (B) positive ion of D-Leu and L-Leu in fragment of 900 Da, (C) positive ion of D-Leu control, (D) positive ion of L-Leu control, (E) positive ion of D-Glu in fragment of 900 Da, (F) positive ion of D-Glu control.

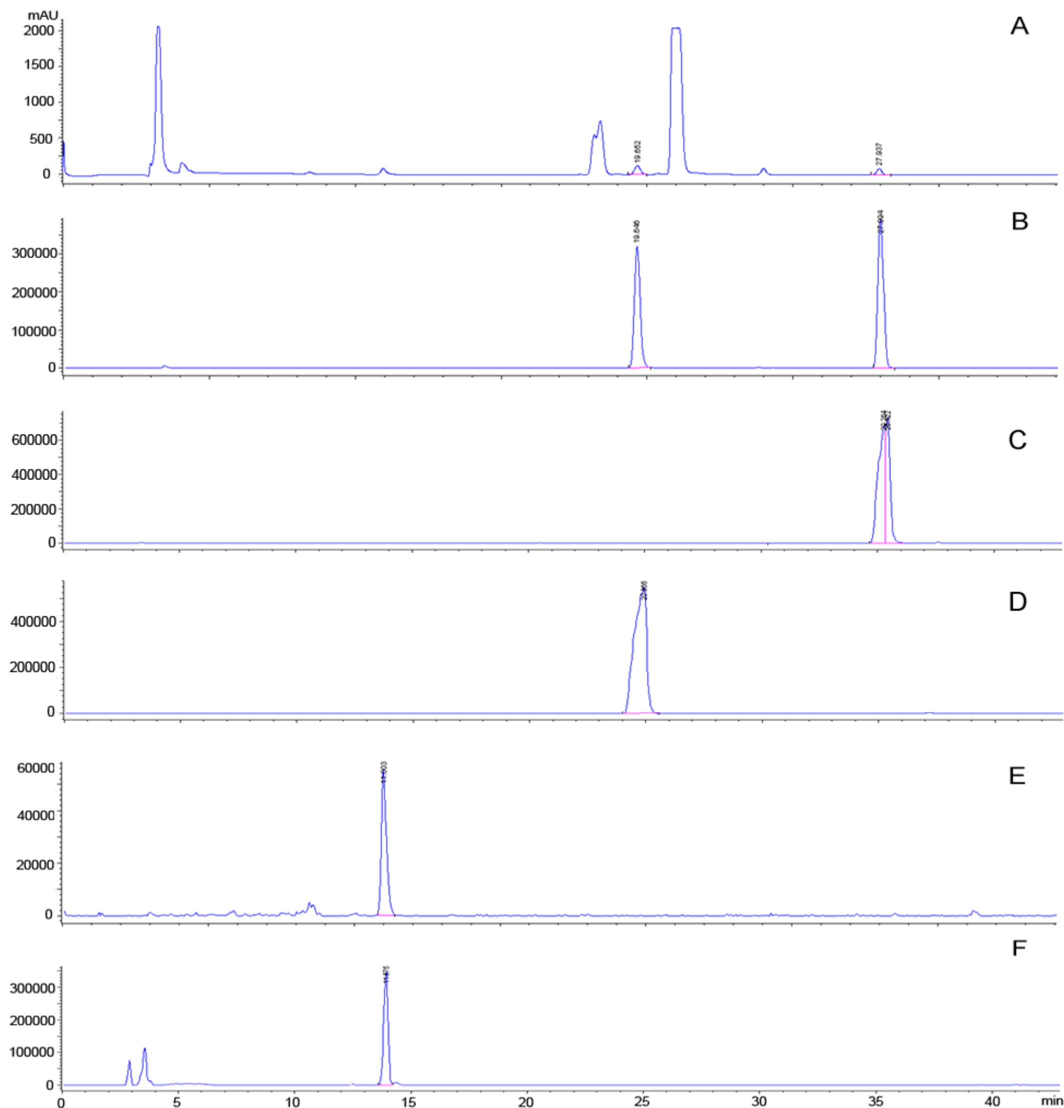

Figure S13. Marfey analysis of a fragment of 900 Da from partial hydrolysis. (A) Fragment of 900 Da MS, (B) positive ion of D-Leu and L-Leu in fragment of 900 Da, (C) positive ion of D-Leu control, (D) positive ion of L-Leu control, (E) positive ion of D-Glu in fragment of 900 Da, (F) positive ion of D-Glu control.

Table S1. Isolate species capable of inhibiting DOT-T1E-18 growth.

Genus	Isolate	Closest neighbour	Similarity (%)	Source
Pseudomonas	260O	*Pseudomonas* sp.	100	Olive soil
	215TR	*P. nitroreducens*	99	Tinto River
	165W	*P. alcaligenes*	100	Waste water treatment plant
	179W	*P. pseudoalcaligenes*	99	Waste water treatment plant
	178W	*P. aeruginosa*	100	Waste water treatment plant
	188W	*P. stutzeri*	100	Waste water treatment plant
	216O	*P. mendocina*	99	Olive soil
	250J	*Pseudomonas* sp. strain 250J	100	EEZ Garden
	254J	*P. pecoglossicida*	99	EEZ Garden
	231PS	*P. putida*	99	Punta del Sebo
	217J	*P. cuatrocienegasensis*	99	EEZ Garden
	265PS	*P. monteilli*	99	Punta del Sebo
Bacillus				
	146TR	*Bacillus* sp.	99	Tinto River
	278TR	*B. pumilus*	99	Tinto River
	263TR	*B. cereus*	99	Tinto River
	195TR	*B. megaterium*	99	Tinto River
	270TR	*B. thuringiensis*	99	Tinto River
	279MT	*B. subtilis*	99	Muelle del Tinto
	176W	*B. amyloquefaciens*	99	Waste water treatment plant
	187W	*B. safensis*	99	Waste water treatment plant
Lysinibacillus				
	234PS	*Lysinibacillus* sp.	99	Punta del Sebo
	249MT	*L. fusiformis*	99	Muelle del Tinto
Shewanella				
	240TR	*Shewanella* sp.	99	Tinto River
	241PS	*S. algae*	99	Punta del Sebo
Alishewanella				
	255W	*Alishewanella* sp.	98	Waste water treatment plant
Vibrio				
	225TR	*V. proteoliticus*	99	Tinto River
Acinetobacter				
	230MT	*A. radioresistens*	100	Muelle del Tinto
Rhodococcus				
	144TR	*R. erythropyla*	99	Tinto River
Alcaligenes				

	173W	*A. faecalis*	99	Waste water treatment plant
Aeromonas				
	182J	*A.hydrophila*	99	EEZ Garden
	167O	*A. media*	99	Olive soil
Cupravidus				
	236MT	*Cupravidus* sp.	96	Muelle del Tinto
Raoultella				
	208W	*R. planticola*	99	Waste water treatment plant
Enterobacter				
	207TR	*Enterobacter* sp.	99	Tinto River
	212J	*E. aerogenes*	97	EEZ Garden

Table S2. Antimicrobial compounds found in the 255W culture extracts. Positions correspond with the numbers that appear in Figure S3.

Position	Compound	Molecular Formula	Molecular Weight (g mol^{-1})
1	Plipastatin A1	$C_{72}H_{110}N_{12}O_{20}$	1463.94
2	Plipastatin A2	$C_{73}H_{112}N_{12}O_{20}$	1477.73
3	Plipastatin B1	$C_{74}H_{114}N_{12}O_{20}$	1491.76
4	Plipastatin B2	$C_{75}H_{116}N_{12}O_{20}$	1505.79
5	4-L-Alaninesurfactin C1	$C_{51}H_{89}N_{7}O_{13}$	1008.29
6	7-L-Valinesurfactin C1	$C_{52}H_{91}N_{7}O_{13}$	1022.31
7	Surfactin A o C1	$C_{53}H_{93}N_{7}O_{13}$	1036.34

Table S3. Antimicrobial compounds found in the 249MT extract. Positions correspond with the numbers that appear in Figure S4. NPB – not produced by bacteria.

Position	Compound	Molecular Formula	Molecular Weight (g mol^{-1})
1	NPB (Erythrolic acid D)	$C_{19}H_{24}O_5$	332.39
2	NPB	$C_{18}H_{22}O_4$	302.36
3	NPB	$C_{16}H_{16}O_4$	272.29
4	NPB	$C_{18}H_{18}O_4$	298.33
5	NPB	$C_{18}H_{22}O_3$	286.36

Table S4. Antimicrobial compounds found in the 225TR extract. Positions correspond with the numbers that appear in Figure S5. No DNP – not described in database of natural products.

Position	Compound	Molecular Formula	Molecular Weight (g mol^{-1})
1	Serratomolide C	$C_{28}H_{50}N_2O_8$	542.70
2	4-deoxy thiomarinol H	$C_{30}H_{50}N_2O_8$	566.72
3	No DNP	$C_{30}H_{52}N_2O_8$	568.74
4	No DNP	$C_{30}H_{54}N_2O_8$	570.75

Table S5. Antimicrobial compounds found in the 250J extract. Positions correspond with the numbers that appear in Figure 1.

Position	Compound	Molecular Formula	Molecular Weight (g mol^{-1})
1	Xantholysin D	$C_{84}H_{146}N_{18}O_{23}$	1775.09
2	Xantholysin B	$C_{83}H_{144}N_{18}O_{23}$	1761.04
3	Xantholysin A	$C_{84}H_{146}N_{18}O_{23}$	1775.08
4	Xantholysin C	$C_{86}H_{148}N_{18}O_{23}$	1802.0

Chapter 5

Draft whole-genome sequence of the antibiotic-producing soil isolated *Pseudomonas* sp. strain 250J

Molina-Santiago, C., Udaondo, Z., and Ramos, J.L. (2014). Draft Whole-Genome Sequence of the Antibiotic-Producing Soil Isolate *Pseudomonas* sp. strain 250J. Under review at Environmental Microbiology Reports.

Summary

Bacteria of the genus *Pseudomonas* are becoming increasing well known for their ability to produce a wide range of antimicrobial compounds. In a large scale screening for antibiotic producers, we identified *Pseudomonas* sp. strain 250J, a soil isolate that uses 4-hydroxyphenylacetate as the sole carbon source which produces cyclic lipodepsipeptides of the xantholysin family during the stationary phase of growth. The closest relatives of this strain are *P. mosselii, P. soli* and *P. entomophila.* Sequencing of the 250J genome allowed us to find the genes relevant to antibiotic production, those which allow utilization of 4-hydroxyphenylacetate as a sole carbon source and a set of genes potentially involved in pathogenicity for this isolate.

Introduction

Strains of the genus *Pseudomonas* are known to be able to colonize a wide range of ecological niches due to their capacity to adapt and survive to different environmental conditions (Dos Santos *et al.,* 2004). These microorganisms generally contain relatively large genomes (about 6 Mb) and their core genome is in the range of 3500 genes (Udaondo *et al.,* unpublished). It has been found that the core genes of the genus *Pseudomonas* define its basic physiological properties regarding the transport of a broad range of chemicals, central carbon metabolism, nitrogen and sulfur assimilation, alternative respiratory chains and chemotaxis. In addition, *Pseudomonas* strains have a set of expansive "accessory genes" that contribute to the different strain-specific properties, such as bioremediation of pollutants, biocontrol, and pathogenicity of humans or plants (Silby *et al.,* 2011, Wu *et al.*, 2011).

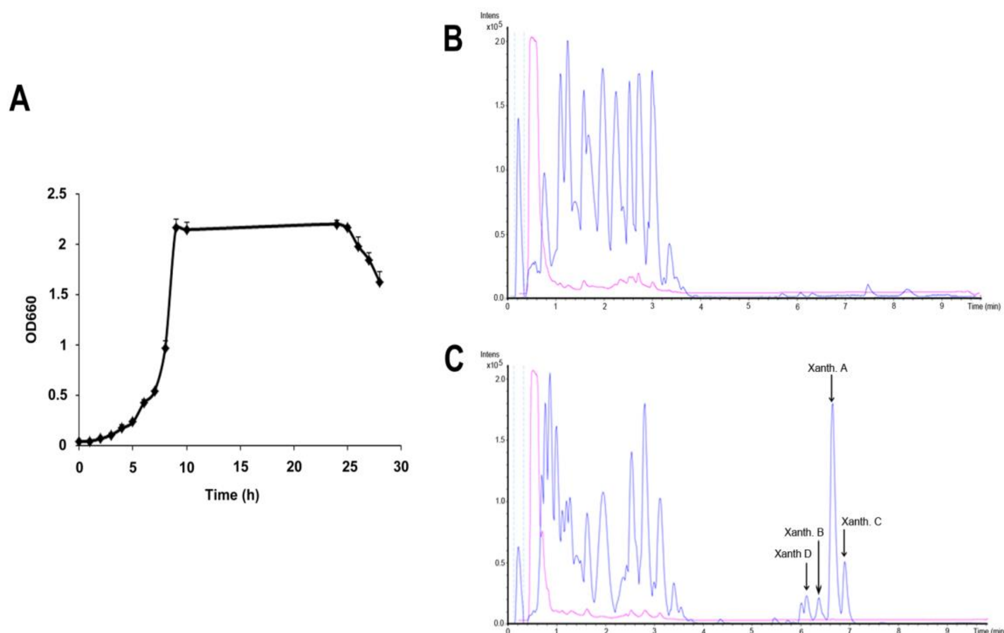

Figure 1. Growth curve of *Pseudomonas* sp. 250J growing on LB (A); and xantholysin production in exponential phase (B), and in stationary phase (C). Xantholysin compounds are indicated in the chromatogram. Pink line indicates UV signal and blue line indicates ionized masses. The high pink peak at the beginning of the chromatogram is due to the DMSO used to dissolve the supernatant, which is not retained in the column.

Results and discussion

Pseudomonas sp. strain 250J is a Gram-negative, aerobic, soil bacterium we isolated in M9 minimal medium (Abril *et al.,* 1989) using 4-hydroxyphenylacetate as the sole carbon source. Utilization of 4-hydroxyphenylacetate is restricted to a limited number of *Pseudomonas* strains and, therefore, this metabolism is not part of the "core" physiology of all *Pseudomonas* strains. We found that strain 250J is able to produce antibiotic compounds that inhibit growth of Gram-positive and Gram-negative microorganisms. These chemicals were then identified as cyclic lipodepsipeptides of the xantholysins family (Figure 1). It is known that xantholysin A has antibacterial, antifungal and antitumoral activity (Li *et al.,* 2013).

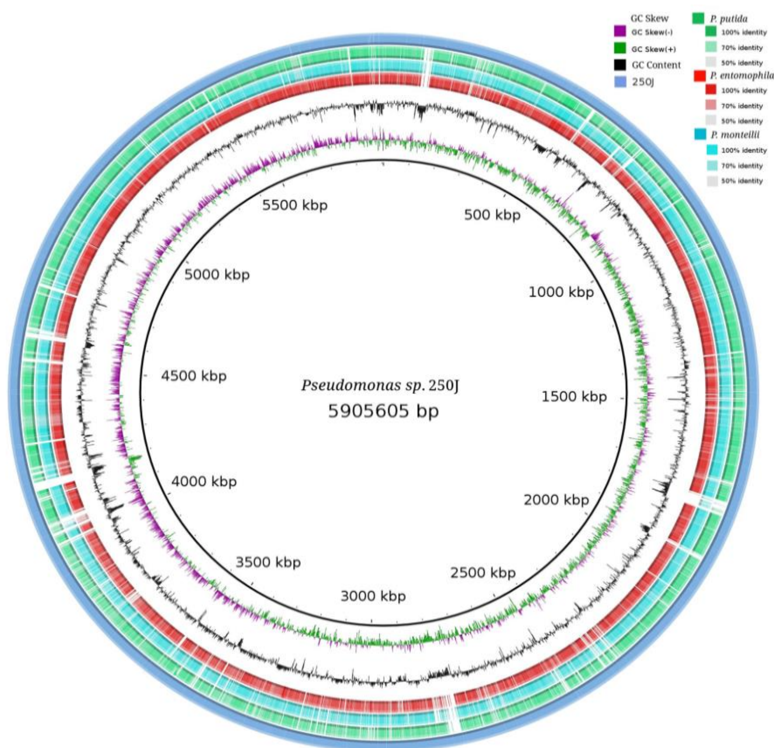

Figure 2. BLAST comparison of draft genome of *Pseudomonas* sp. 250J against *Pseudomonas entomophila*, *Pseudomonas monteilii* and *Pseudomonas putida* KT2440 using BRIG. The innermost rings depict the GC content (Black) and GC Skew (purple/green) rings of Pseudomonas sp. 250J, followed by the the query sequences of *P. entomophila* (red), *P. monteilii* (light blue), *P. putida* KT2440 (green) and *Pseudomonas* sp. 250J (blue) colored according to BLAST identity.

To further study the utilization of 4-hydroxyphenylacetate as a carbon source, and determine the genes involved in the production of xantholysin, we decided to sequence the genome of 250J strain. Genomic DNA containing the chromosome was purified from *Pseudomonas* sp. strain 250J using the Wizard® Genomic DNA Purification Kit. Whole-genome sequencing was performed using 150-base, paired-end reads on the Illumina MiSeq Platform at the basic biology service of the University of Granada (Spain). A total of 2 x 6,838,152 paired sequences were generated and subsequently analyzed and checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The genomic sequence of *Pseudomonas* sp. strain 250J was obtained by assembly of the two paired-end data sets using Velvet (1.2.10 version) (Zerbino and Birney, 2008) with parameters determined by Velvet Optimizer 2.2.5 (http://bioinformatics.net.au/software.velvetoptimiser.shtml) resulting in 219 contigs with N_{50} of 62 Mbp and a read depth of 50X. The draft whole-genome sequence of *Pseudomonas* sp. strain 250J is made up of a 62.20%-GC circular chromosome of 5,906,591 bp with no plasmid (GenBank accession no. JHEE01000000). This genome encodes 5,226 putative genes, including 9 rRNA and 73 tRNA genes that were annotated with the NCBI Prokaryotic Genome Annotation Pipeline and RAST server (Aziz *et al.,* 2008). We show a circular representation of the 250J genome in Figure 2 where we represent the ordered draft genome generated with the BRIG software and the percent identity that it shares with other *Pseudomonas* species such as *P. entomophila*, *P. monteilii* and *P. putida* KT2440.

The 4-hydroxyphenylacetate degradation pathway involves the conversion of this aromatic compound to homoprotocatechuate, which subsequently undergoes ring cleavage to eventually yield succinate and pyruvate, as shown in Figure 3A. In *P. entomophila* and *P. putida* U the set of genes encoding the pathway enzymes are grouped in a cluster (*hpaCBXIHFDEG2G1AR*) (Vodovar *et al.,* 2006, Arcos *et al.,* 2010), while in 250J the pathway genes for degradation of 4-hydroxyphenylacetate are distributed in three unlinked operons. The genes present in contig 89 corresponded to the *hpaIHFDEG2G1AR* cluster as in *P. fluorescens* SBW25 (Paliwal *et al.,* 2014) (Figure 3B), while the genes *hpaBC*, that encode the 4-hydroxyphenylacetate 3-hydroxylase that performs the first reaction of the pathway, and a potential 4-hydroxyphenylacetic acid transporter, *hpaX*, were found in contigs 90 and 110, respectively.

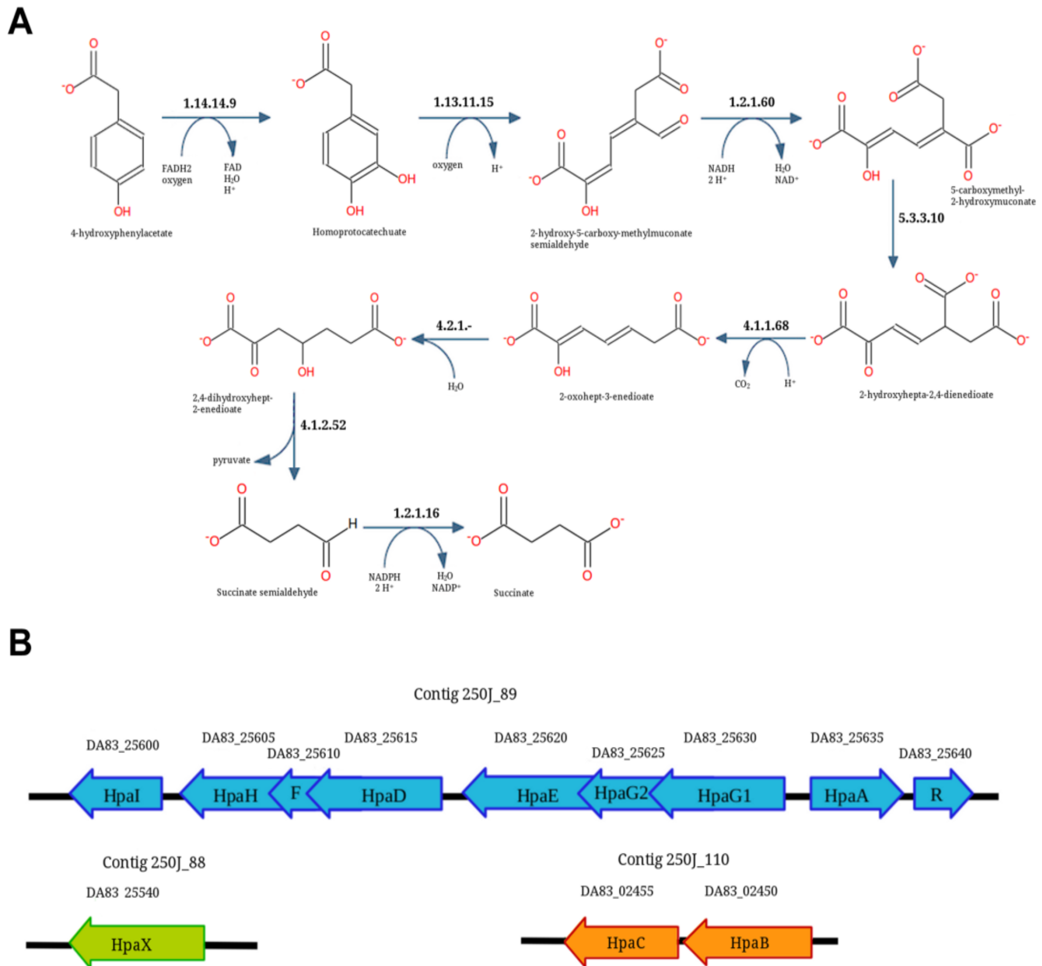

Figure 3. Pathway and genes related with 4-hydroxyphenylacetate degradation. (A) Degradation pathway of 4-hydroxyphenylacetate. (B) Genes related with the synthesis of enzymes that are involved in the degradation pathway.

It is known that cyclic lipodepsipeptides are generally produced by nonribosomal peptides synthetase (NRPS) gene clusters. By using the antiSMASH software for rapid identification, annotation, and analysis of secondary metabolite biosynthesis genes (Blin *et al.,* 2013), we were able to predict 24 PKS/NRPS gene clusters for putative biosynthetic secondary metabolites. These clusters can be organized into three groups that correspond to three gene clusters specifically: three clusters involved in bacteriocin biosynthesis, nine nonribosomal peptide synthetases (NRPS), and twelve clusters predicted as putative NRPS of unknown

function. Close analysis of the nine NRPS allowed identification of five clusters that are involved in the biosynthesis of four different xantholysins. These four cyclic lipodepsipeptides have been identified in the culture supernatant of *Pseudomonas* sp. strain 250J and their molecular masses and chemical formula have been determined (Figure 1C): xantholysin A (1775.08 Da, $C_{84}H_{146}N_{18}O_{23}$), xantholysin B (1761.07 Da, $C_{83}H_{144}N_{18}O_{23}$), xantholysin C (1802.0, $C_{86}H_{148}N_{18}O_{23}$), and xantholysin D (1775.09 Da, $C_{84}H_{146}N_{18}O_{23}$), being xantholysin A the majoritarian one. The high number of PKS/NRPS sequences that could be producing secondary metabolites related with antimicrobial, antifungal and insecticidal compounds demonstrate the great armory that this strain present to defend against other microorganisms and that could use to colonize other niches.

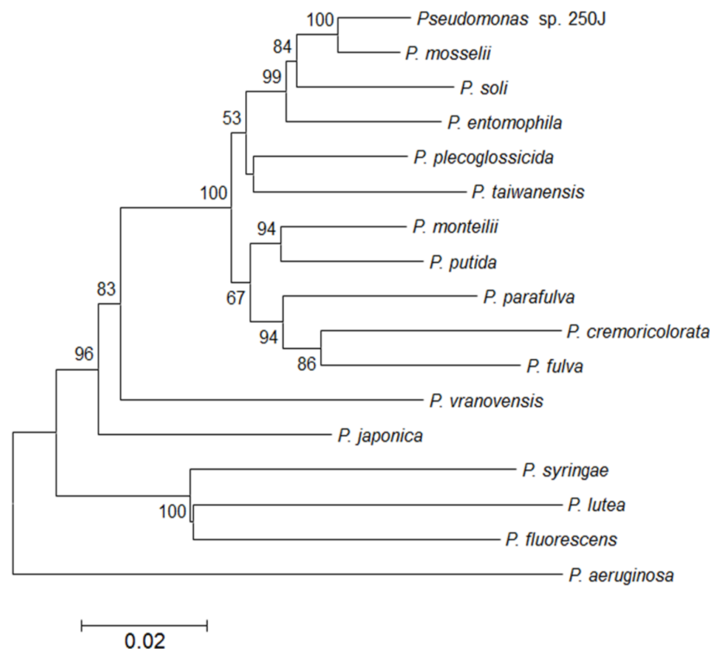

Figure 4. Neighbour-joining tree illustrating the phylogenetic position of strain *Pseudomonas* sp. 250J and related members of the genus *Pseudomonas* based on partial concatenated gene sequences. Bar, 0.01 expected nucleotide substitution per site. *Pseudomonas aeruginosa* was used as outgroup. Only bootstrap values above 50% are indicated (1000 resamplings) at branchings.

BLAST analyses were then done to pinpoint the *xtlA, xtlB, xtlC* genes and the transcriptional regulator gene *xtl*R, which are related with xantholysin production in *P. putida* BW11M1. These genes are also conserved in *Pseudomonas* sp. 250J genome sequence (92, 94, 95 and 97 percent of identity sequence, respectively). The xantholysin transporter is encoded by the *xtlD, xtlE* and *xtlF* genes and exhibits a percent of sequence identity with those of BW11M1 of 97, 94 and 94 respectively. The xantholysin genes are organized in two clusters, one made of *xtlF, xtl*R and *xtlA*, and the other one performed by the *xtlD, xtlE, xtlB* and *xtlC* genes.

MLSA analysis comparison of the 250J strain with numerous other *Pseudomonas* strains revealed that the closest relatives to 250J are *P. mosselii, P. soli* and *P. entomophila*. *Pseudomonas* sp. 250J forms a monophyletic cluster with *P. mosselii, P. soli* and *P. entomophila* (Figure 4). Furthermore, Eztaxon analysis of 16S rRNA genes supported the results obtained by MLSA analysis. *Pseudomonas entomophila* is known as a microorganism with insecticidal activity and having the ability to kill a number of other invertebrates. A comparison between the genomes of *P. entomophila* and 250J was carried out in order to identify genes potentially related to pathogenicity. We found a number of proteases and toxins (Table 2), such as AprA (DA83_19105), an alkaline protease that is encoded together with its secretion system and involved in virulence in many species (Miyoshi and Shinoda, 2000); serine-proteases (DA83_13385 and DA83_13390); or the RTX toxin (DA83_05900). We tested the hemolytic activity of 250J and confirmed that this strain was able to produce hemolysis of goat blood. Strain 250J bears the *hcnABC* genes (DA83_11195, DA83_11200, DA83_11205) that are related with hydrogen cyanide production, a toxic compound required for the virulence of *P. entomophila* against *Drosophila melanogaster*, killing of *Caenorhabditis elegans* by *P. aeruginosa* (Gallagher and Manoil, 2001) and in the suppression of soil-borne plant pathogens by certain *P. fluorescens* species (Haas and Defago, 2005). Protease and lipase activities together with hydrogen cyanide production were confirmed experimentally, what suggest the possible use of this strain in plague biocontrol. The genome of the 250J strain does not bear homologous of genes encoding for the insecticidal complex (TcdA, TcdB and TccC), which is present and characteristic of all the entomophatogens.

In summary, the present results show that the soil isolate *Pseudomonas* sp. strain 250J is a strain capable of naturally overproducing xantholysins and is able to use

4-hydroxyphenylacetic acid as a carbon source. Although strain 250J lacks known insecticidal genes, it is closely related to *P. entomophila* and bears a set of genes potentially involved in pathogenicity.

Table 1. Genes related to virulence factors in *Pseudomonas* sp. 250J.

***Pseudomonas* sp. 250J gene ID NAME**	***P. entomophila* gene ID NAME**	**% IDENT**	**PROTEIN NAME**
DA83_06635	pen:PSEEN0817	100	Putative ATP-dependent protease
DA83_09325	pen:PSEEN0378	100	Carboxy-terminal processing protease (EC 3.4.21.102)
DA83_22155	pen:PSEEN1868	100	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92) (Endopeptidase Clp)
DA83_22160	pen:PSEEN1869	100	ATP-dependent Clp protease ATP-binding subunit ClpX
DA83_22165	pen:PSEEN1870	100	Lon protease (EC 3.4.21.53) (ATP-dependent protease La)
DA83_17100	pen:PSEEN1266	100	Putative glycoprotein endopeptidase metalloprotease (EC 3.4.-.-)
DA83_05900	pen:PSEEN3925	100	Putative RTX toxin
DA83_05910	pen:PSEEN3928	100	Putative multidrug/toxin efflux protein, ATP binding and membrane protein
DA83_19720	pen:PSEEN3450	100	Response regulator GacA (Global antibiotic and cyanide control protein, LuxR/UhpA family)
DA83_13285	pen:PSEEN4521	100	HtrA-like serine protease AlgW
DA83_13385	pen:PSEEN3028	100	Putative subtilisin-like serine protease (EC 3.4.21.-)
DA83_13390	pen:PSEEN3027	100	Putative serine protease PspB (EC 3.4.21.-)
DA83_12475	pen:PSEEN4646	100	Putative RelB protein (Antitoxin)
DA83_03315	pen:PSEEN2207	100	ATP-dependent Clp protease, ATP-binding subunit ClpA
DA83_03320	pen:PSEEN2206	100	ATP-dependent Clp protease adaptor protein clpS
DA83_09030	pen:PSEEN5063	100	ATP-dependent protease subunit HslV (EC 3.4.25.2)
DA83_09035	pen:PSEEN5064	100	ATP-dependent protease ATPase subunit HslU (Unfoldase HslU)

DA83_13785	pen:PSEEN1143	100	Putative protease C56
DA83_14315	pen:PSEEN1431	100	Putative carboxy-terminal protease for penicillin-binding protein 3 Prc (EC 3.4.21.102)
DA83_14345	pen:PSEEN4293	100	Serine protease MucD
DA83_14410	pen:PSEEN4367	100	Lon protease (EC 3.4.21.53)
DA83_21355	pen:PSEEN3499	100	Putative protease, U32 family
DA83_08020	pen:PSEEN4875	100	Putative virulence-associated protein MvpT
DA83_19100	pen:PSEEN1550	100	Alkaline metalloprotease AprA (EC 3.4.24.40)
DA83_19105	pen:PSEEN1551	100	Inhibitor of protease AprA
DA83_19110	pen:PSEEN1552	100	Alkaline protease (AprA) secretion protein AprD (EC 3.6.3.-)
DA83_19115	pen:PSEEN1553	100	Alkaline protease (AprA) secretion protein AprE
DA83_19120	pen:PSEEN1554	100	Alkaline protease (AprA) secretion protein AprF
DA83_07205	pen:PSEEN0787	100	ATP-dependent zinc metalloprotease FtsH (EC 3.4.24.-)
DA83_12070	pen:PSEEN4678	100	ATP-dependent Clp protease, ATP-binding subunit ClpB
DA83_12260	pen:PSEEN0100	100	Putative metalloprotease, M16 family (EC 3.4.-.-)
DA83_07880	pen:PSEEN4834	100	Putative ATP-dependent protease La domain protein
DA83_17275	pen:PSEEN4213	100	Putative membrane-associated Zn-dependent proteases 1
DA83_26220	pen:PSEEN3077	100	Putative clp protease (Protease subunit)
DA83_10440	pen:PSEEN0145	100	Putative type I toxin efflux membrane fusion protein
DA83_10445	pen:PSEEN0144	100	Putative type I toxin efflux ATP-binding protein (EC 3.6.3.-)
DA83_10450	pen:PSEEN0143	100	Putative type I toxin efflux outer membrane protein TolC

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number JHEE00000000. The version described in this paper is version JHEE01000000.

Acknowledgments

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IV. GENERAL DISCUSSION

Prior to this PhD work, *Pseudomonas putida* DOT-T1E had been isolated from a wastewater treatment plant in Granada and identified as a solvent-tolerant microorganism (Ramos *et al.*, 1995). This strain has been studied in Prof. Ramos' group as a paradigm of solvent tolerance (reviewed in Ramos *et al.*, 2002 and 2015). Mutant generation using a Km-mini-Tn5 transposon and a selection of solvent-sensitive mutants allowed the identification of three efflux pumps, called TtgABC, TtgGHI and TtgDEF, as relevant components of solvent tolerance in *P. putida* DOT-T1E (Ramos *et al.*, 1998; Rojas *et al.*, 2001; Segura *et al.*, 2012). In parallel, the group also showed that cross-resistance occurred between solvent tolerance and antibiotic resistance (Daniels and Ramos, 2009). A number of assays revealed that TtgABC is the main efflux pump responsible for the extrusion of antimicrobials, such as tetracycline, ampicillin or chloramphenicol (Terán *et al.*, 2003); whereas TtgDEF and TtgGHI play a major role in solvent removal (Rodríguez-Herva *et al.*, 2007). Although TtgDEF does not seem to be involved in antibiotic resistance, TtgGHI was shown to play a secondary role in antibiotic extrusion (Rojas *et al.*, 2003; Molina-Santiago *et al.*, 2014a).

The TtgR and TtgV repressors modulate the expression of TtgABC and TtgGHI, respectively. The *ttgDEF* operon is under the control of both TtgV and a second repressor called TtgT (Terán *et al.*, 2007). All these repressors are released from their cognate operators in the presence of effectors leading to a higher level of transcription (Terán *et al.*, 2003; Guazzaroni *et al.*, 2004; Fillet *et al.*, 2009). TtgV and TtgT both recognize a range of mono- and bi-aromatic compounds, which leads to the induction of the TtgGHI and TtgDEF pumps, allowing the cells to extrude a wide range of aromatic hydrocarbons and lineal alcohols in addition to a number of antibiotics such as ampicillin, tetracycline, chloramphenicol, piperacillin, etc. The TtgV regulator has been crystallyzed (Lu *et al.*, 2010) and found to be made up of two domains linked by a long α-helix. The domain located at the C-terminal end is able to bind ligands such as 1-naphthol, benzonitrile and indole (Guazzaroni *et al.*, 2007), while the other domain exhibits an HTH motif responsible for DNA binding. The structure of the TtgV regulator also bound to its DNA operator was also solved (Lu *et al.*, 2010). The analysis of the co-crystal identified a set of specific interactions between TtgV and its operator mediated by S35, R47, T49, and R52. TtgV is a tetramer that, when bound to DNA, is re-organized in two asymmetric dimers. Residues in a loop between positions 129 and 131, and I136 are of critical importance for this re-organization. On the other

hand, residues E102 and R98 play an important role in communication between the two domains as well as Q86. Comparison of crystallized TtgV in its free apo-form and bound to DNA showed a 90º break at the Q86 residue (Fillet *et al.*, 2009; Lu *et al.*, 2010).

In this thesis, our aim was to further assess the role of Q86, R19, S35, G44 and P46 in greater detail. The substitution of the glutamine located at position 86 by other amino acids, such as asparagine, provoked a change in the way the protein unfolds and an increase in affinity to ligands. We reasoned that the central role of Q86 could be its involvement in the establishment of two intradimer hydrogen bonds (that are not formed with substituent amino acids) and which are broken when DNA binding occurs producing the kink observed in the crystallized TtgV bound to DNA (Lu *et al.*, 2010; Molina-Santiago *et al.*, 2014b).

Mutations at positions 19 and 35, located outside of the HTH motif, prevented these mutant variants from binding to target DNA. This is a quite unique event in transcriptional regulators since key residues are those that are part of the HTH. These residues are, therefore, likely to play a role not only in binding, but also in the DNA bending trait exhibited by TtgV, especially when it is known that TtgV binding to DNA not only prevents the access of the RNA polymerase to the promoter, but also causes a bent that impedes contacts. Alanine substitutions at positions 44 and 46 near the HTH showed an increase in ligand affinity and thermal stabilization of the protein, but these replacements produced different responses. The G44A mutant was unable to bind to DNA, in contrast with P46A (Molina-Santiago *et al.*, 2014b). Multiple alignments of these amino acids at these positions in the IclR family indicated conservation of S35 and R19, what suggest that these amino acid residues could be related with a general role in the repression mechanism of members of this family. However, Q86 is not a conserved residue in the family and could play a specific role in TtgV as a repressor.

As it was mentioned above, TtgV is the transcriptional regulator of the TtgGHI efflux pump (Guazzaroni *et al.*, 2007), which is involved in the extrusion of solvents. We decided to study its role in bactericidal and bacteriostatic antibiotic extrusion using mutants in different RND efflux pumps. As we expected, a strain deficient in TtgABC was more sensitive to antibiotics than the wild-type strain and T1E-PS28 (a TtgGHI mutant). A double mutant in both efflux pumps was even more sensitive to antibiotics than the strain mutated only

in TtgABC, highlighting the secondary role of the TtgGHI efflux pump in antibiotic extrusion (Molina-Santiago *et al.*, 2014a). Kohanski *et al.* (2007) suggested that bactericidal antibiotics kill bacteria through the generation of oxygen-free radicals. While this paper published in Cell was generally accepted by scientific community and has been cited repetitively, a series of results by Keren *et al.* (2013), Liu and Imlay (2013), and Mahoney and Silhavy (2013) refuted the hypothesis put forward by Kohanski *et al.* (2007), whose proposal was based on the fact that bactericidal compounds quenched HPF fluorescence through the generation of free hydroxyl radicals. Our transcriptomic studies in *P. putida* demonstrated that bactericidal antibiotics, such as ampicillin, do not induce an oxidative stress response, and also support the relation between quenching of HPF and bactericidal compounds; although the molecular bases of these observations were unknown. Two recent studies have pointed out a putative relation between cell size and HPF fluorescence (Renggli *et al.*, 2013; Paulander *et al.*, 2014). The authors suggest that an increase in cell size provoked by the action of many bactericidal antibiotics leads to an increase in the HPF signal. This explanation is based on the observation that bactericidal antibiotics, such as β-lactams and fluoroquinolones, alter cell morphology increasing cell size due to an increment in the filamentation without cell division. This effect is not observed when cells are treated with bacteriostatic antibiotics because these compounds do not induce filamentation or an increase in cell size.

Intra- and interspecies signaling molecules usually regulate the expression and phenotypes of eukaryotic and prokaryotic cells due to their interaction with different regulatory proteins. Indole is considered as an intraspecies signaling molecule since it influences processes such as drug resistance, plasmid stability, virulence and biofilm formation (Hirakawa *et al.*, 2005; Lee *et al.*, 2007; Chu *et al.*, 2012). Indole can also act as an interspecies signal producing effects such as growth inhibition of *Aspergillus niger* generated by the indole excreted by *E. coli*, or the increase of drug resistance in *Salmonella enterica* (Vega *et al.*, 2013); and even as an interkingdom signaling molecule as it plays a role in the inhibition of colonization of the human gastrointestinal tract by *E. coli* (Bansal *et al.*, 2007). The characterization of the profile of TtgV effectors identified indole as one of the molecules recognized by this transcriptional regulator (Guazzaroni *et al.*, 2007). In this thesis we have studied the transcriptomic response of *P. putida* DOT-T1E to indole and we have found that indole, as a signaling molecule, changes the

expression of a number of genes related to different functions. This transcriptomic study has also showed that indole increases the expression of TtgV leading to the up-regulation of the TtgGHI efflux pump and increasing the antibiotic resistance in a DOT-T1E strain mutated in TtgABC (Molina-Santiago *et al.*, 2014a). The results obtained suggested a role of the TtgGHI efflux pump in bacterial communication since its induction by the indole synthesized and excreted to the medium by other microorganisms provokes a response in DOT-T1E, what permits them to adapt and survive to environmental changes. It is critical to understand the mechanisms underlying bacterial communication and bacterial resistance because of its importance in the context of antibiotic treatments and infections produced by multiple microbes.

Transcriptomic analyses performed with high-throughput molecular technologies and bioinformatics have supported much information about the different responses and defense mechanisms of *P. putida* DOT-T1E against a wide range of antibiotics belonging to families with different mechanisms of action. The results obtained are in concordance with the described mechanisms of action of the antibiotics used and have also added some new information. Comparative analyses of genes over-expressed in each condition showed that many genes appeared to be up- or down-regulated in response to different stresses, whereas the over-expression of the largest groups of genes is response-specific. Functional comparisons clearly demonstrated that functions related with binding, catalytic activities and metabolic processes are important no matter the condition under study and despite the fact that only few of these over-expressed genes actually appeared to be the same in all the tested conditions. The above set of results, obtained with RNA-sequencing, demonstrated that the mechanisms of action of antibiotics lead to different transcriptional responses, inducing or repressing the expression of a set of genes against the antibiotics tested, plus a number of non-specific genes. The high number of hypothetical genes and the over-expression of sRNAs in response to different antibiotics, with an *a priori* no clear relation, highlights that our knowledge on antibiotic resistance response is still far from being complete.

In our studies, we have discovered 138 new sRNAs in DOT-T1E, which dramatically increases the 16 already described for this strain. Conservation analyses of these sRNAs have shown that 30 of them are unique for DOT-T1E which could be related with specific functions in this bacterium, while 33% of

them belong to the core-genome of *Pseudomonas putida* (Udaondo and Ramos, unpublished) and point towards a potential role in the regulation of central functions. Expression profiles in the presence of the tested antibiotics showed a relation with the profiles obtained with mRNAs, thus suggesting the probable implication of sRNAs in antibiotic resistance, as has also been pointed out in *Staphylococcus aureus* (Howden *et al.*, 2013). In any case, more studies will be needed to complete our understanding of the regulatory role of these elements in the antibiotic resistance in *P. putida*.

Today, the problem of persistent bacteria and multi-resistant strains that provoke chronic infections is arising and the cocktail of antimicrobials is not enough to combat them since the rate of antimicrobials discovery is lower than the appearance of new resistances or the acquisition of resistance mechanisms by multidrug-resistant bacteria. Nearly all antibiotics in use today, or their derivatives, were discovered during the 1940s to 1960s, which calls on the need to discover new drugs (Lewis, 2013). Historically, the first platform created for the discovery of new antimicrobials was developed by Waksman in the 1940s which used soil-derived Streptomycetes that produced a growth inhibition halo around a susceptible microorganism. After that, different strategies were used to discover new antimicrobials, such as 'classic' screening methods, chemical modification of known antimicrobials, potentiation of activity of known compounds used in combination with 'enhancers', searching of new targets and inhibitors of virulence and pathogenesis (Moellering, 2011).

The critical situation inspired us to use a mutant *P. putida* DOT-T1E strain, antibiotic-sensitive, deficient in TtgABC efflux pump, and a collection of 2400 isolates obtained from different places to develop a biotechnological application to discover new antibiotics. In spite of the fact that a TtgABC mutant strain is more sensitive to antibiotics than the wild-type, this mutant is still able to grow in the presence of considerable amounts of antibiotics. The high basal antibiotic resistance level of this mutant strain led us to identify compounds with a relative high antibiotic activity against Gram-negative bacteria. We used a method based on inhibitory growth assays for the selection of producer strains and, after that, HPLC-MS analyses determined the presence of new antibiotic compounds. We found strains that produced antibiotics to which DOT-T1E TtgABC mutant was sensitive, thus confirming the screening method. We also identified a new *Pseudomonas* strain called *Pseudomonas* sp. 250J, closely related to *P. entomophila, P. soli*

and *P. mosselii* according to 16S rRNA and MLSA analyses, with the ability to produce cyclic lipodepsipeptides of the, until the date of discovery, new xantholysin family.

Pseudomonas sp. strain 250J was isolated from the soil at Estación Experimental del Zaidín, in Granada, using 4-hydroxyphenylacetic acid as carbon source. The analysis of the genome sequence provided us more information about the xantholysin synthesis pathway and other elements of interest. For instance, we found the genes responsible of 4-hydroxyphenylacetic acid degradation located in three different contigs separated in the genome. This organization has also been found in other *Pseudomonas* strains, such as *P. fluorescens* or *P. entomophila*. Genome comparison with *P. entomophila* showed the presence of many virulence factors that could be related with insect infection, though critical insecticidal toxic complexes are not present in 250J strain; therefore, *in vivo* assays will be needed to clarify its insecticidal virulence. Other bioinformatics analyses focused on the search for genes related with the synthesis of secondary metabolites, where genes related with antibiotic synthesis could be found. Twenty four PKS/NRPS gene clusters related with the synthesis of bacteriocins, xantholysin and nonribosomal peptide synthetases were found. These results show a wide range of compounds and virulence factors that could be part of the defense and attack armory of 250J for niche colonization.

As was mentioned above, *Pseudomonas* sp. strain 250J is able to produce 4 compounds of the xantholysin family. These compounds are cyclic lipodepsipeptides that present a ring composed of 8 amino acids and a lipidic tail. In the course of this investigation, Li *et al.* (2013) published a paper describing the synthesis pathway, structure and antimicrobial and antifungal properties of xantholysin A, the main compound of the Xantholysin family, by *Pseudomonas putida* BW11M1. These authors concluded that Xantholysin A has antimicrobial activity against Gram-positive bacteria, but also a small effect against many Gram-negative ones, antifungal activity, a role in biofilm formation in *P. putida* BW11M1, and an effect in surface colonization (Li *et al.*, 2013).

We used the 250J strain to study its antimicrobial properties and we were able to isolate and purify xantholysin A and xantholysin C which allowed us to study their inhibitory activities and their synergistic effect in combination with classic antibiotics. Our results confirm the antimicrobial activity of xantholysin A against Gram-positive bacteria with MIC values of 25-50 μg ml^{-1} while its activity against

Pseudomonas strains was much lower (500-1000 μg ml^{-1}). However, xantholysin C showed similar effects against Gram-positive bacteria but, on the other hand, it showed higher activity against *Pseudomonas* strains (62.5-125 μg ml^{-1}). These results suggest specific activity of each of the xantholysin compounds produced by the 250J strain, which demonstrates a wide range of different responses against different bacteria showing an armory of defense mechanisms.

Taken together, the results in this thesis have contributed to the better understanding of the mechanisms of antibiotic resistance in *Pseudomonas putida* DOT-T1E, focusing on the secondary efflux pump related with antibiotic extrusion, TtgGHI, as well as on its transcriptional regulator TtgV. We have deciphered the role of key amino acids of TtgV in DNA binding and signal transmission and we have analyzed the role of the signaling molecule indole in resistance to bactericidal and bacteriostatic antibiotics. We have demonstrated that indole acts increasing antibiotic resistance of *P. putida* DOT-T1E through the induction of the TtgGHI efflux pump and that this compound is an interspecies signaling molecule as seen from the effect of the indole produced by *E. coli* in DOT-T1E. Transcriptomic studies of DOT-T1E in the presence of different antibiotics have confirmed the mechanisms of action of the tested compounds and have highlighted new genes related with the antimicrobial defense of bacteria. We have also described the presence of 138 new sRNAs, which have critically increased the 16 sRNAs already described in this strain. These sRNAs have shown expression profiles similar to those obtained with mRNAs, which suggests that sRNAs could also have a role in the regulation of antibiotic resistance in DOT-T1E. This thesis ends with the biotechnological application of a mutant DOT-T1E strain as a reporter for a new platform for the discovery of new antimicrobials, describing a new *Pseudomonas* sp. strain able to produce xantholysins with antimicrobial properties. Furthermore, the combination with classical antibiotics increases their antimicrobial activity and points towards possible applications of xantholysin compounds in infective treatments.

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V. CONCLUSIONS/CONCLUSIONES

The results obtained in this thesis have led us to the following conclusions:

1. The TtgGHI efflux pump is involved in the extrusion of aromatic hydrocarbons and has secondary activity for the extrusion of antibiotics. In this thesis we show that the role of TtgGHI as an antibiotic efflux pump is particularly evident in a mutant background devoid of the TtgABC pump, the most relevant RND pump in antibiotic extrusion.

2. The transcription of the *ttgGHI* operon is known to be controlled by the TtgV regulator, whose 3D structure in apo-form and co-crystallized with its cognate promoter has been resolved. TtgV is a two domain protein and these domains are interconnected by an extended linker. The 3D structure revealed that a kink occurs in TtgV at residue Q86 when it is bound to DNA (Lu *et al.*, 2010, *Genes Dev*). We have shown that Q86 is a key residue in the intramolecular communication that occurs between the ligand binding domain and the HTH binding domain. Mutations at this position that elicit a 90º kink prevent contact between TtgV and DNA. To explain this, we hypothesize that when Q86 is replaced by another amino acid, two intra-dimer hydrogen bonds are broken, which strongly compromises the ability of TtgV to contact DNA.

3. Mutations at positions 19, 35 and 44, located outside of the canonical HTH DNA binding domain, prevent the binding of TtgV to DNA, revealing that these amino acids are important for TtgV-DNA contact.

4. Indole is known to be an effector of TtgV (Guazzaroni *et al.,* 2007, *J. Mol. Biol.*). While indole is not produced by *Pseudomonas putida*, when it is supplied exogenously it induces the expression of the *ttgGHI* operon and, in turn, improves the resistance of DOT-T1E to a wide range of bactericidal and bacteriostatic compounds.

5. Indole, which serves as an interspecies signal for *Pseudomonas*, induces a wide range of genes, including the TtgGHI pump as well as regulatory proteins such as TtgV, genes related with amino acid metabolism,

inorganic ion metabolism, energy production and carbohydrate metabolism, metal transport systems and oxygen stress related genes.

6. RNA-Seq transcriptomic analyses confirmed the mechanisms of action attributed to ciprofloxacin, tetracycline, gentamicin, spectinomycin, ampicillin, chloramphenicol, kanamycin and rifampicin; but also revealed other genes that are modulated in response to these compounds. These genes include a large number of overexpressed hypothetical genes that lack a clear function, demonstrating that our understanding of the bacterial antibiotic response is far from complete.

7. Transcriptomic analyses revealed the presence of 138 new sRNAs in *P. putida* DOT-T1E, dramatically increasing the 16 that have been already detected in previous studies. The expression of these sRNAs in the presence of antibiotics suggests that they play a role in the regulation of antibiotic resistance.

8. The modification of the *P. putida* DOT-T1E strain through mutation of TtgABC (DOT-T1E-18) has created of a new platform for the discovery of new antimicrobial compounds. DOT-T1E-18 is more sensitive to antibiotics than the wild-type strain, a property that enabled us to accurately identify new antimicrobial-producing bacteria.

9. The platform described above has led to the isolation of the *Pseudomonas* sp. strain 250J. This strain is able to produce a family of xantholysin compounds. Studies that involved the isolation and purification of xantholysin A and xantholysin C have demonstrated that these compounds act as antimicrobials and are able to prevent growth of Gram-positive and Gram-negative bacteria. These compounds also increase the activity of other antibiotics when used in combination, showing that they possess synergistic antibiotic effects against a range of different bacteria.

Los resultados obtenidos en esta Tesis Doctoral nos han llevado a las siguientes conclusiones:

1. La bomba de extrusión TtgGHI se encarga de la expulsión de hidrocarburos aromáticos al exterior celular, y muestra como actividad secundaria la extrusión de antibióticos. En esta Tesis se muestra que el papel de TtgGHI como una bomba de expulsión de antibióticos es particularmente evidente en un mutante el cual carece de la bomba TtgABC, la bomba de tipo RND más relevante en la extrusión de antibióticos en *P. putida* DOT-T1E.

2. Estudios previos demostraron que la transcripción del operón *ttgGHI* está mediada por el regulador TtgV, cuya estructura 3D en su forma libre y co-cristalizada con el promotor al que reconoce se había resuelto previamente (Lu et al., 2010, *Genes Dev*). TtgV es una proteína formada por dos dominios los cuales están interconectados por una extensa α-hélice. La estructura 3D revela que TtgV presenta un pliegue en el residuo Q86 cuando se encuentra unido a ADN. En esta Tesis Doctoral se muestra que Q86 es un residuo clave en la comunicación entre el dominio de unión a ligando y el dominio HTH, ya que mutaciones en esta posición impiden el pliegue de 90º, lo que evita el contacto entre TtgV y el ADN. Una probable explicación de su importancia es que cuando Q86 se reemplaza por otros aminoácidos, se rompen los dos puentes de hidrógeno intradímero que se forman en condiciones normales, lo que previene el contacto proteína-ADN.

3. Mutaciones en las posiciones 19, 35 y 44 localizadas fuera del dominio HTH de unión a ADN evitan la unión de TtgV a ADN, lo que muestra un papel importante de estos aminoácidos en el contacto TtgV-DNA.

4. El indol, un compuesto que no producen las cepas de *Pseudomonas*, es un efector de TtgV que cuando se suministra de forma exógena induce la expresión del operón *ttgGHI*. Ello conlleva un incremento en la expresión de TtgGHI, lo cuál se traduce en un aumento en la resistencia de DOT-T1E a un amplio rango de compuestos bactericidas y bacteriostáticos.

5. El indol es una señal interespecie para *Pseudomonas* ya que un amplio rango de genes se ven inducidos en respuesta a indol, incluyendo no solo la bomba TtgGHI sino también genes relacionados con el metabolismo de aminoácidos, el metabolismo de iones inorgánicos, la producción de energía, el metabolismo de carbohidratos, diferentes sistemas de transporte de metales y genes relacionados con el estrés oxidativo.

6. Análisis transcriptómicos realizados con secuenciación masiva de ARN confirman los mecanismos de acción asociados a los antibióticos ciprofloxacino, tetraciclina, gentamicina, espectinomicina, ampicilina, cloranfenicol, kanamicina y rifampicin; pero también muestran otros genes relacionados con la respuesta bacteriana a estos compuestos. Un alto número de genes con función hipotética también aparecen sobre expresados, lo que demuestra que aún estamos lejos de comprender por completo la respuesta bacteriana a antibióticos.

7. Análisis transcriptómicos han revelado la presencia de 138 nuevos sRNA en *P. putida* DOT-T1E, lo que incrementa los 16 detectados en estudios previos. La expresión de estos sRNA en presencia a antibióticos sugiere que podrían desarrollar un papel en la regulación de la resistencia a antibióticos.

8. El uso de una cepa de *P. putida* DOT-T1E mutada en TtgABC (DOT-T1E-18) como cepa control nos ha permitido crear una nueva plataforma para el descubrimiento de nuevos compuestos antimicrobianos. DOT-T1E-18 es más sensible a antibióticos que la cepa silvestre, lo que permite identificar bacterias productoras de antibióticos de una forma más sensible.

9. La plataforma para el descubrimiento de nuevos compuestos antimicrobianos ha permitido aislar la cepa *Pseudomonas* sp. 250J. Esta cepa es capaz de producir cuatro compuestos de la familia de las xantolisinas. El aislamiento y purificación de xantolisina A y xantolisina C ha demostrado el efecto de estos compuestos como antimicrobianos ya que son capaces de evitar el crecimiento de bacterias Gram-positivas y

Gram-negativas. Estos compuestos, además, incrementan la actividad de otros antibióticos cuando se ensayan conjuntamente, lo que demuestra un claro efecto sinérgico frente a diferentes bacterias.