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"Análisis Funcional de Genes Presentes en el Plásmido pUM505 de *Pseudomonas aeruginosa*"

Tesis

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I. RESUMEN

En las bacterias la transferencia horizontal de genes es realizada por elementos genéticos móviles como los plásmidos. El plásmido conjugativo pUM505 se aisló de una cepa clínica de *Pseudomonas aeruginosa* obtenida de un paciente hospitalizado, y se determinó que es capaz de conferir resistencia a cromato y mercurio. El objetivo de este trabajo fue realizar la secuenciación y análisis de la secuencia del plásmido pUM505, caracterizar la función de los genes *umuD* y *umuC* (*umuDC*), y analizar la estructura y función del gen *pdi*. La secuenciación y el análisis de la secuencia de nucleótidos del plásmido pUM505, determinó que este replicón contiene 138 regiones codificantes. pUM505 posee dos regiones, la primera corresponde a una isla de resistencia a metales pesados, con genes que codifican proteínas implicadas en la resistencia a cromato y mercurio; la segunda región de pUM505 corresponde a una isla genómica que muestra homología con genes presentes en la isla de patogenicidad. Esta isla posee genes que codifican proteínas implicadas en la replicación y transferencia del plásmido, así como proteínas probablemente involucradas en virulencia.

Adicionalmente el plásmido pUM505 contiene el operón *umuDC* que codifica proteínas similares a la DNA polimerasa V propensa a error. El gen *umuC* aparentemente está truncado y su producto probablemente no es funcional. El gen *umuD* se renombró *umuDpR* (por *umuD* *p*lasmid *R*egulator), éste posee una caja SOS traslapada con el probable promotor reconocido por el factor sigma-70; mediante fusiones transcripcionales se demostró que el promotor del gen *umuDpR* es activado por Mitomicina C, agente generador de daño al DNA. UmuDpR mostró

23% de identidad con LexA de *P. aeruginosa*, represor de la respuesta SOS. Se determinó, mediante ensayos de qRT-PCR, que la proteína UmuDpR reprime la expresión de los genes SOS de *P. aeruginosa* controlados por LexA. Sin embargo, mediante los ensayos de cambio de movilidad electroforética, se encontró que UmuDpR no se une a la región reguladora de los genes SOS, sugiriendo un mecanismo indirecto de regulación.

pUM505 también posee el gen *pdi*, que se predice que codifica a una disulfuro isomerasa de proteínas (Pdi), enzima que cataliza la formación de enlaces disulfuro entre residuos de cisteína en proteínas. El gen *pdi* posee en su región reguladora un posible promotor reconocido por el factor sigma S. Mediante fusiones transcripcionales se determinó que el promotor del gen *pdi* es funcional, con una mayor expresión en la fase estacionaria, y ésta es regulada de manera dependiente del factor sigma S, por lo que la proteína Pdi de pUM505 podría requerirse para cambios adaptativos que ocurren en esta etapa del crecimiento bacteriano. Mediante un análisis *in silico* de la proteína predicha Pdi de pUM505 se determinó que posee un dominio estructural conocido como plegamiento tiorredoxina, con dos motivos conservados: el sitio activo $C_{66}XXC_{69}$ y el motivo “asa cis-prolina”, típicos de las oxidorreductasas DsbAs de las γ-proteobacterias. Pdi también posee un par de cisteínas conservadas (C_{111} y C_{157}) propias de la subclase α-DsbA de las α-proteobacterias. Además, Pdi posee un residuo conservado treonina (T_{183}) característico de las disulfuro isomerasas (DsbC/DsbG) de *Escherichia coli*. Por lo tanto, al tener Pdi podría tener la función de oxidación/reducción e isomerización de enlaces disulfuro en específico de *P. aruginosa*.

II. ABSTRACT

In bacteria horizontal gene transfer is mediated by mobile elements such as plasmids. The conjugative pUM505 plasmid was isolated from a clinical strain of *Pseudomonas aeruginosa* and confers resistance to chromate and mercury. The aims of this work were to realize the sequencing and sequence analysis of pUM505 plasmid, characterize the function of *umuD* and *umuC* (*umuDC*) genes, and analyze the structure and function of *pdi* gene. The sequence analysis of pUM505 showed that this plasmid contains 138 coding regions and presents two well-defined regions, the first one corresponds to a genomic island, which possesses the chromate and mercury resistance genes; the second region corresponds to a genomic island of pathogenicity. This island, in addition to replication and conjugative transfer genes, has genes probably involved in virulence.

Additionally, pUM505 contains the *umuDC* operon that encodes proteins similar to error-prone repair DNA polymerase V. The *umuC* gene appears to be truncated and its product is probably not functional. The *umuD* gene, renamed *umuDpR* (by *umuD* *p*lasmid *R*egulator), possesses an SOS box overlapped with a Sigma factor 70-type promoter; accordingly, transcriptional fusions revealed that the *umuDpR* gene promoter is activated by Mitomycin C (MMC). The predicted sequence of the UmuDpR protein displays 23% identity with the *P. aeruginosa* SOS-response LexA repressor. Through Reverse transcription-quantitative PCR (qRT-PCR) assays was determined that the UmuDpR protein is a repressor of *P. aeruginosa* SOS genes controlled by LexA. Electrophoretic mobility shift assays, however, did not show binding of UmuDpR to 5' regions of SOS genes, suggesting an indirect mechanism of regulation.

The plasmid pUM505 contains the *pdi* gene that encodes a protein similar to putative protein disulfide isomerases (Pdi), enzymes that catalyze formation of protein disulfide bonds. The putative regulatory region of the *pdi* gene possesses a potential promoter related with S type sigma factor. Transcriptional fusions of this region revealed that the *pdi* gene promoter is functional, with maximal expression in the stationary growth phase, and this is dependent on the S-type sigma factor. Therefore, pUM505 Pdi protein may be required for adaptive changes that occur during this stage of bacterial growth. An *in silico* analysis of predicted protein Pdi showed that this has a structural "thioredoxin fold" domain with two conserved motifs: $C_{66}XXC_{69}$, a catalytic site, and the "cis-proline loop" motif, present in oxidoreductases DsbAs from γ-proteobacteria. Also, Pdi has two conserved cysteines (C_{111} and C_{157}), typical of the subclase α-DsbA present in α-proteobacteria. Furthermore, Pdi possesses a conserved threonine residue (T_{183}) characteristic of disulfide isomerases such as (DsbC/DsbG) from *Escherichia coli*. Therefore, Pdi could have the function of oxidation/reduction and isomerization of disulfide bonds of proteins from *P. aeruginosa*.

Key words: disulfide bonds, LexA, *Pseudomonas*, pUM505 plasmid, SOS response.

III. INTRODUCCIÓN

1. Género *Pseudomonas*

Pseudomonas literalmente significa “falsa unidad”, derivado del griego *pseudo* (ψευδο “falso”) y *monas* (μονάς/μονάδα “una sola unidad”). El término “monada” se usaba en la microbiología antigua para nombrar a los organismos unicelulares. Inicialmente en la historia de la microbiología el término *Pseudomonas* fue empleado como sinónimo de gérmenes. El género *Pseudomonas* fue descrito por primera vez por Migula (1894) y es uno de los géneros bacterianos más diversos y ubicuos, cuyas especies se han aislado desde la Antártida hasta el Trópico y están presentes en sedimentos, muestras clínicas, especímenes como plantas, hongos y animales, agua dulce, suelo, rizósfera de plantas, mares y desiertos, entre otros (Peix *et al.* 2009).

La importancia de este género no es sólo en términos de su capacidad de causar enfermedades en plantas y animales, incluyendo a los humanos, y a su destreza en la biodegradación y remoción de un gran número de compuestos naturales y sintéticos, sino también por su utilidad como un sistema de estudio de rutas metabólicas y en el análisis de la expresión de genes (Özen y Ussery 2012).

1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa es una bacteria Gram-negativa, móvil y mesófila que se encuentra en una gran variedad de nichos ecológicos incluyendo agua, suelo, plantas, así como humanos y animales (Battle *et al.* 2009). Esta bacteria posee numerosos factores de virulencia que contribuyen a su patogénesis (Strateva y Mitov 2011). Adicionalmente, *P. aeruginosa* posee una resistencia intrínseca a diversos

antibióticos debido a la barrera que representa la membrana externa y a la presencia de transportadores de expulsión de drogas de la membrana interna (Poole 2011).

El genoma central de *P. aeruginosa* se define como los genes que están presentes en casi todas las cepas de esta bacteria cuyos genomas han sido secuenciados y éstos codifican a un grupo de factores metabólicos y patogénicos que se comparten por todas estas cepas, independiente del origen (ambiental o clínico) (Kung *et al*. 2010). El genoma central de *P. aeruginosa* constituye aproximadamente el 90% del total del genoma y está altamente conservado entre cepa y cepa; sin embargo, los genes accesorios, presentes en plásmidos o islas genómicas, han contribuido a la evolución del genoma de este microorganismo (Kung *et al*. 2010).

2. Plásmidos

Los plásmidos son moléculas de DNA extracromosómico circular o lineal, que codifican funciones no esenciales, cuya replicación es independiente del cromosoma del hospedero. Actualmente, se han reportado más de 4600 secuencias completas de plásmidos de bacterias, arqueas y eucariotas (Revisado por Shintani *et al.* 2015). Los plásmidos están presentes en casi todas las especies bacterianas y el tamaño de éstos es variable (Frost *et al*. 2005; Slater *et al.* 2008). El plásmido pRKU1 de *Thermotoga petrophila* RKU1, con 846 pares de bases (pb) posee solo un gen implicado en la replicación (*rep*), es considerado el plásmido más pequeño; por otra parte, el plásmido pGMI1000MP de *Ralstonia solanacearum* GMI1000 con 1,674 kilobases (kb) es reportado como el plásmido de mayor tamaño (Smillie *et al.* 2010), el tamaño de éste es un 10 y 2.8 veces más grande que el genoma de la bacteria simbionte *Carsonella ruddii* y la bacteria parásita *Mycoplasma genitalium*,

consideradas la primera y segunda bacteria con los genomas más pequeños, respectivamente.

En la naturaleza, los plásmidos incrementan la diversidad genética y promueven la adaptación bacteriana por la transferencia horizontal de genes, proceso que introduce información genética no parental dentro de una célula. Algunos plásmidos pueden transferirse a otras bacterias por conjugación. Smillie *et al.* (2010) reportaron que alrededor del 14% del total de los plásmidos totalmente secuenciados se predice que pueden ser conjugativos, siendo la conjugación uno de los mecanismos más efectivos para la propagación de elementos genéticos entre las bacterias (Revisado por Shintani *et al*. 2015).

El primer plásmido que se aisló y caracterizó fue en Japón a finales de los 50´s y se relacionó con la adquisición de nuevos genes de resistencia a antibióticos (Revisado por Watanabe 1963). Los plásmidos se han estudiado exhaustivamente tanto por sus propiedades genéticas como fenotípicas, incluyendo resistencia a antibióticos y metales pesados, degradación de compuestos xenobióticos, determinantes de virulencia, producción de bacteriocinas, resistencia a la radiación e incremento de la frecuencia de mutación (Molbak *et al.* 2003). Éstas son llamadas “funciones accesorias”, que facilitan la rápida adaptación a la presión selectiva de un ambiente nuevo o transitorio (Levin y Bergstrom 2000).

En los primeros años del análisis de los plásmidos se determinó diversas propiedades de éstos que incluyen el tamaño, la incompatibilidad, la replicación y la transferencia de genes que éstos poseen (Novick 1969). El conocimiento de las relaciones entre las características de los plásmidos y la taxonomía del hospedero es importante con la finalidad de comprender cómo los plásmidos se han propagado

entre los microorganismos (Shintani *et al.* 2015). Los plásmidos se utilizan como vectores de clonación en ingeniería genética por su capacidad de replicarse de manera independiente del DNA cromosómico, así como también porque es relativamente fácil manipularlos e insertar nuevas secuencias.

3. Islas genómicas

Muchos genes accesorios adquiridos por transferencia horizontal forman bloques sinténicos que son conocidos como islas genómicas (IGs), que comúnmente tienen entre 10 a 200 kb. Estas islas difieren del contenido de nucleótidos de guanina y citosina (GC) del resto del cromosoma y generalmente están localizadas junto a genes que codifican para tRNAs que actúan como sitios de integración; así mismo, las IG son flanqueadas por secuencias de repetidos invertidos (RI) y en ocasiones contienen genes que codifican integrasas o factores relacionados a sistemas de conjugación de plásmidos involucrados en la transferencia de las IGs (Darmon y Leach 2014). De acuerdo al tipo de genes, las IGs pueden nombrarse como islas de patogenicidad, de simbiosis, metabólicas, de resistencia a antibióticos o mercurio (Juhas *et al.* 2009; Darmon y Leach 2014).

Las IG son segmentos de DNA discretos, presentes en cepas cercanamente relacionadas, cuya importancia actual es debida a su contribución a la diversificación y adaptación de los microorganismos; teniendo así un impacto significativo en la plasticidad genómica y en la evolución de los microorganismos; así como en la diseminación de genes involucrados en resistencia a antibióticos, en la virulencia y en rutas catabólicas (Juhas *et al.* 2009).

Se ha demostrado que algunas IGs son capaces de realizar su propia movilización, ésta puede llevar a cabo la escisión de su localización en el cromosoma y

reintegrarse en el cromosoma de un nuevo hospedero como IG o en forma de plásmido (Juhas *et al*. 2009).

3.1. Islas de patogenicidad

Cuando las IGs contienen genes que codifican determinantes de virulencia, como toxinas, invasinas, adhesinas, proteínas efectoras, etc., se denominan como islas de patogenicidad (PAIs) (Dobrindt *et al.* 2004), este concepto se acuñó por primera vez a finales de los 80´s por Hacker y colaboradores, quienes investigaron las bases genéticas de la virulencia de aislados uropatógenos de *Escherichia coli* (Hacker *et al*. 1990).

Un ejemplo bien descrito de PAIs que contribuye a la variación de la virulencia en cepas de *P. aeruginosa* es la familia de las islas cromosómicas que contienen al gen *exoU* (Kulasekara *et al*. 2006), que codifica a la proteína ExoU, una potente citotoxina con actividad de fosfolipasa A2 necesaria para la virulencia en *P. aeruginosa* (Finck-Barbancon *et al*. 1997; Hauser *et al.* 1998; Sato *et al*. 2003).

Así mismo, se identificó la isla cromosómica PAGI-1 considerada la primer PAI presente en aislados clínicos de *P. aeruginosa*, la cual está ausente en el genoma de la cepa de referencia *P. aeruginosa* PAO1 aun cuando esta cepa también fue aislada de una fuente clínica (Liang *et al.* 2001). Otras PAIs identificadas en el cromosoma son las islas PAPI-1 y PAPI-2, las cuales están presentes en la cepa *P. aeruginosa* PA14 un aislado clínico altamente virulento con respecto a *P. aeruginosa* PAO1 (He *et al.* 2004). Sin embargo, existe el elemento móvil pKLC102, identificado en un aislado clínico de *P. aeruginosa*, que puede permanecer como IG integrada en el cromosoma o bien como un plásmido (Klockgether *et al*. 2004).

4. Plásmido pUM505

El plásmido conjugativo pUM505 se aisló de una cepa clínica de *P. aeruginosa* obtenida de un paciente hospitalizado, y se determinó que es capaz de conferir resistencia a cromato y mercurio (Cervantes *et al.* 1990). El primer mecanismo descrito de resistencia bacteriana a cromato es el conferido por el gen *chrA* codificado en pUM505 (Alvarez *et al.* 1999) y es el mejor caracterizado en bacterias (Ramírez-Díaz *et al.* 2008). La secuenciación preliminar del plásmido pUM505 (derivada de un primer ensamblaje de la secuencia de nucleótidos) y el análisis de ésta permitió identificar regiones codificantes que probablemente están relacionadas con replicación, el mantenimiento, transferencia del plásmido, así como propiedades adaptativas (Fig. 1).

Se determinó que el gen *chrA* de pUM505 se encuentra formando parte de un probable operón *chrBAC*, el cual está situado dentro de una posible región de transposición que está a su vez flanqueada por los genes *tnp*A, que codifica una transposasa/resolvasa, y *tnp*R, que codifica una resolvasa/integrasa (Fig. 1). La presencia de operones con arreglo génico similar se han identificado en el plásmido pMOL28 de la bacteria Gram-negativa *Cupriavidus metallidurans* (Juhnke *et al.* 2002), en un transposón de la bacteria aeróbica estricta Gram-negativa *Ochrobactrum tritici* (Branco *et al.* 2008) y en el megaplásmido de la bacteria aeróbica Gram-negativa *Burkholderia xenovorans* LB400 (Acosta-Navarrete *et al.* 2014).

El plásmido pUM505 contiene dos probables operones de resistencia a mercurio (*mer*): un operón completo, constituido por los genes *merRTPFADE*, y un operón

incompleto, formado solamente por los genes *merRTP* (Fig. 1). Se ha descrito que los genes que codifican proteínas que confieren resistencia a mercurio (Hg^{2+}) se

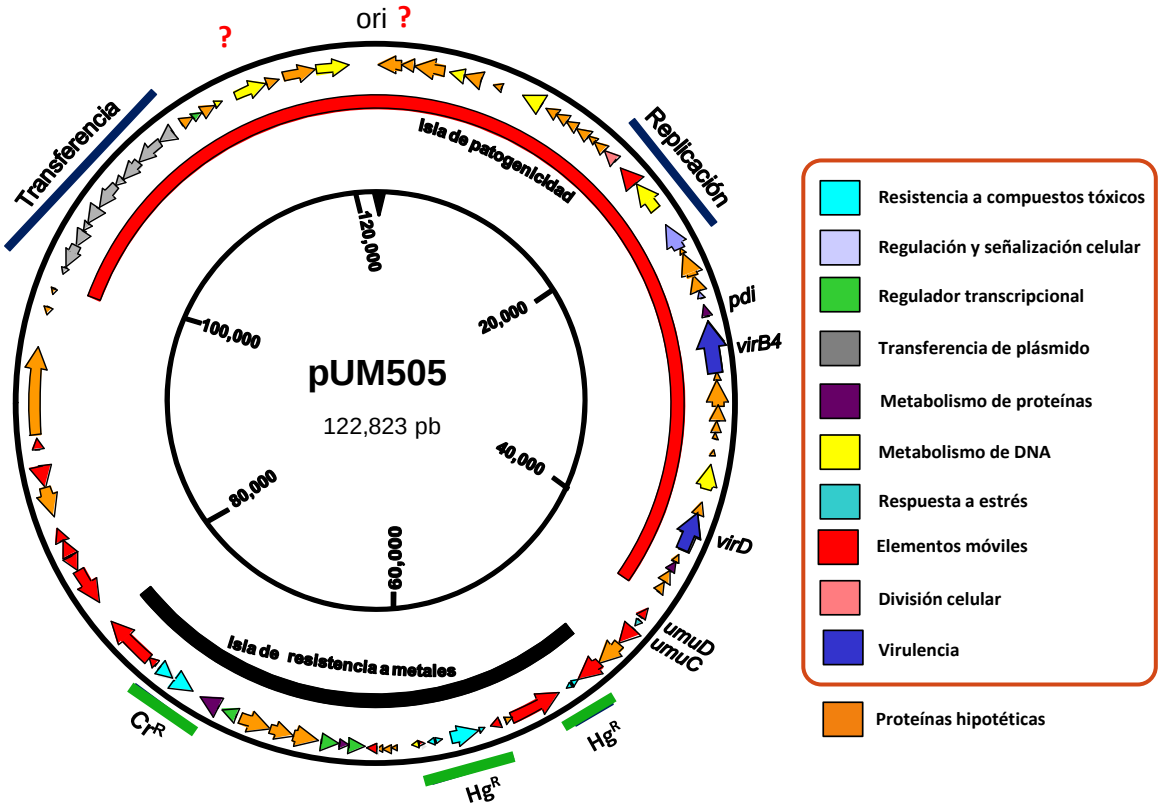

Figura 1. **Mapa genético preliminar del plásmido pUM505**. Las regiones codificantes se muestran por flechas o puntas de flecha indicando la dirección de la transcripción. La posible función de las proteínas se indica en el recuadro de la derecha. Las principales regiones del plásmido son: **1)** Isla de patogenicidad (barra roja), la cual posee genes relacionados con transferencia conjugativa, replicación, virulencia y tolerancia a estrés; **2)** Isla de resistencia a metales pesados (barra negra), que posee los genes que participan en la resistencia a cromato (Cr^R) y mercurio (Hg^R).

encuentran localizados en cromosomas, plásmidos y transposones en una gran diversidad de arreglos, a menudo con la duplicación y distribución de genes *mer* entre varios replicones en una misma célula (Barkay *et al.* 2003).

Adicionalmente, un análisis de la secuencia de pUM505 con el programa BLAST mostró que éste posee genes homólogos a los genes *virB4*/*virD4* involucrados en la virulencia, los cuales no se encuentran formando parte del mismo operón, a diferencia de sus homólogos de *Agrobacterium tumefaciens*; por el contrario, cada uno forma parte de un probable operón cuyos miembros no han sido aún caracterizados.

Así mismo, pUM505 tiene los genes *umuD* y *umuC* (*umuDC*), cuyos productos son similares a las subunidades de la DNA polimerasa V propensa a error; estos genes forman parte de la respuesta SOS, que se describió por primera vez en *E. coli* (Walker 1984), la cual comprende más de 40 genes (denominados el regulón SOS) involucrados en la reparación de DNA, sistemas implicados en la replicación de DNA propensos a error y en la regulación de la división celular (Revisado por Patel *et al*. 2010).

Los genes SOS son regulados negativamente por la proteína represora LexA, que reconoce específicamente un motivo regulador (la caja SOS) presente en la región promotora de estos genes (Fernández de Henestrosa *et al.* 2003). La inducción de los genes SOS inicia por la activación de la proteína RecA, que se une a los segmentos de DNA de cadena sencilla (ssDNA) generando la formación de filamentos que catalizan la autoproteólisis del represor LexA, ocasionando la activación de los genes SOS (Revisado por Patel *et al*. 2010).

En la mayoría de las bacterias, los genes *umuDC* así como sus homólogos *mucAB*, *rumAB* y *rulAB,* son comúnmente adyacentes y sus regiones codificantes están traslapadas formando operones (Revisado por Ippoliti *et al.* 2012). La expresión de estos genes responde al daño al DNA, debido a que poseen una caja SOS en su región promotora (Revisado por Kivisaar 2010). La proteína UmuD de *E. coli* es inicialmente sintetizada como un polipéptido de 139 aa, que posteriormente sufre una ruptura en el extremo amino terminal en una reacción dependiente del complejo RecA/ssDNA, originando la forma activa UmuD´ (115 aa) (Revisado por Nohmi 2006). Los productos del gen *umuD* muestran diferentes funciones en la célula. En etapas tempranas de la respuesta SOS en *E. coli*, UmuD forma un complejo con la proteína UmuC ($UmuD_2C$), el cual provoca una pausa en la división celular mientras ocurre la reparación y replicación del DNA llevadas a cabo por otros sistemas (Revidado por Kivisaar 2010). Sin embargo, cuando la respuesta SOS se prolonga, indicando la presencia de un elevado nivel de daño al DNA no reparado, la forma activa UmuD´ se une a UmuC formando la DNA polimerasa V (complejo $UmuD'_2C$; Pol V), la cual lleva a cabo la replicación translesión propensa a error del DNA dañado, proceso conocido como mutagénesis SOS (Revisado por Patel *et al*. 2010).

Existen dos tipos de proteínas parecidas a UmuD: las proteínas UmuD que funcionan en conjunto con UmuC, que regular el ciclo celular y llevan a cabo la síntesis translesión del DNA; y los represores transcripcionales similares a UmuD que actúan de una manera similar que el represor LexA (Hare *et al*. 2006) descritos a continuación.

La bacteria del suelo *Acinetobacter baylyi* ADP1 contiene el operón *umuDAb-umuC* que es inusual en contenido, regulación y función comparado con sus homólogos en

E. coli (Hare *et al.* 2006). Comparado con el producto del gen *umuD* de *E. coli*, el gen *umuDAb* codifica un segmento amino terminal extra de 59 aa, esta región no muestra similitud con ninguna proteína descrita previamente o alguna proteína UmuD estudiada; por otra parte UmuC *A. baylyi* se considera una proteína truncada debido que ésta tiene un tamaño de 50 aa que representa una décima parte de la proteína UmuC de *E. coli* con función reportada de 422 aa, (Hare *et al.* 2006). El operón *umuDAb-umuC* no contiene una caja SOS en su región promotora y su expresión es constitutiva (Hare *et al.* 2006). UmuDAb realiza su autoproteólisis de una manera dependiente de RecA en células expuestas a diversas formas de daño al DNA; UmuDAb posee la pareja de residuos conservados (Ser-Lys) del sitio activo así como la secuencia dipéptida (Ala/Cys)-Gly que se requieren para la autoproteólisis de UmuD de *E. coli*, componente de la Pol V, y del represor LexA de *E. coli*, lo que sugiere que UmuDAb podría tener un mecanismo de auto-proteólisis similar. Sin embargo, los residuos Leu_{101}-Arg_{102} para una autoproteólisis eficiente de la proteína UmuD de *E. coli* no están presentes en UmuDAb, los cuales fueron reemplazados por isoleucina y acido aspártico (posiciones equivalentes 163 y 164); estos dos residuos son más similares a los presentes en los represores transcripcionales de la familia LexA y relacionados (Hare *et al*. 2006). UmuDAb posee 37% de identidad con respecto al represor LexA de *E. coli* éste se limita al C-terminal de la proteína; además no existe similitud entre el dominio N-terminal de unión al DNA de LexA y el de UmuDAb, lo que sugiere un mecanismo indirecto de regulación transcripcional por UmuDAb (Hare *et al*. 2012).

Por otra parte, se ha encontrado que la proteína UmuDAb del patógeno oportunista *Acinetobacter baumannii* ATCC 17978 reconoce secuencias palindrómicas presentes

en los promotores de homólogos *umuDC*, reprimiendo o activando su expresión, por lo que su función es similar a la del represor LexA en otras bacterias. La importancia del papel regulador de UmuDAb es enfatizada por la ausencia de un homólogo LexA en bacterias del género *Acinetobacter* (Aranda *et al.* 2013).

Debido a que la mayoría de las cepas de *P. aeruginosa* carecen de genes *umuDC* cromosomales, y que el operón *umuDC* del plásmido pUM505, posee características similares al regulador UmuDAb de *Acinetobacter*, éste podría funcionar como un regulador de la expresión de genes relacionados con la respuesta de daño al DNA en esta bacteria. El objetivo de este trabajo fue caracterizar la función de los genes *umuDC* de pUM505.

pUM505 también posee el gen *pdi*, que se predice que codifica a una disulfuro isomerasa de proteínas, enzima que en otros organismos cataliza la formación y ruptura de enlaces disulfuro entre residuos de cisteína en proteínas (Chivers *et al.* 1997); dicho gen también será analizado para determinar su función.

El plásmido pUM505 fue secuenciado por segunda ocasión y la secuencia fué ensamblada nuevamente con el propósito de confirmar la presencia de los genes identificados, así como su localización y orientación en el replicón. Sin embargo, una vez obtenida la secuencia final de pUM505 es necesario realizar un análisis detallado de ésta para la elaboración del mapa genético que detalle características de éste, como el origen de replicación, presencia de operones, transposones, islas genómicas y posibles promotores, así como comparar los genes presentes en pUM505 con los reportados en las bases de datos e interpretar la importancia de estos genes.

IV. JUSTIFICACIÓN

Mediante el análisis de la secuencia de nucleótidos preliminar del plásmido pUM505 se encontró que éste posee genes que confieren potencialmente diversas propiedades de adaptación, que en conjunto representarían una ventaja evolutiva para el huésped bacteriano. Sin embargo, es necesario realizar la caracterización de los genes de adaptación identificados en pUM505, como los genes *umuD* y *umuC* que codifican proteínas probablemente involucradas en la respuesta de daño al DNA; Así como el gen *pdi*, que codifica una disulfuro isomerasa de proteínas cuya función está probablemente implicada en la integridad estructural y funcional de otras proteínas. El propósito del análisis de dichos genes es entender cómo este plásmido puede contribuir en la prevalencia, el desarrollo y en la evolución de cepas de *P. aeruginosa* en los distintos ambientes que habita.

V. HIPÓTESIS

El plásmido pUM505 posee los genes *umuD*/*umuC* y el gen *pdi* que codifican proteínas involucradas en la respuesta de daño al DNA y en la formación de enlaces disulfuro en proteínas, respectivamente.

VI. OBJETIVOS

a) Objetivo general

Determinar si los genes *umuD/umuC* y *pdi* del plásmido pUM505 están implicados en la respuesta de daño al DNA y en formación de enlaces disulfuro en proteínas, respectivamente.

b) Objetivos particulares

1. Obtener el mapa genético completo del plásmido pUM505
2. Caracterizar la función de los genes *umuD* y *umuC* de pUM505
3. Establecer la función del gen *pdi* de pUM505.

VI. RESULTADOS

Los resultados generados durante la realización del presente proyecto se presentan en tres capítulos. El capítulo I y II corresponden a los artículos publicados en las revistas internacionales de arbitraje Plasmid (factor de impacto de 1.76) y Microbiology (factor de impacto de 2.83), respectivamente.

CAPÍTULO I.

Nucleotide sequence of *Pseudomonas aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal resistance genes

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Nucleotide sequence of *Pseudomonas aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal resistance genes

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ABSTRACT

We determined the complete nucleotide sequence of conjugative plasmid pUM505 isolated from a clinical strain of *Pseudomonas aeruginosa*. The plasmid had a length of 123,322 bp and contained 138 complete coding regions, including 46% open reading frames encoding hypothetical proteins. pUM505 can be considered a hybrid plasmid because it presents two well-defined regions. The first region corresponded to a larger DNA segment with homology to a pathogenicity island from virulent *Pseudomonas* strains; this island in pUM505 was comprised of genes probably involved in virulence and genes encoding proteins implicated in replication, maintenance and plasmid transfer. Sequence analysis identified *pil* genes encoding a type IV secretion system, establishing pUM505 as a member of the family of IncI1 plasmids. Plasmid pUM505 also contained *virB4/virD4* homologues, which are linked to virulence in other plasmids. The second region, smaller in length, contains inorganic mercury and chromate resistance gene clusters both flanked by putative mobile elements. Although no genes for antibiotic resistance were identified, when pUM505 was transferred to a recipient strain of *P. aeruginosa* it conferred resistance to the fluoroquinolone ciprofloxacin. pUM505 also conferred resistance to the superoxide radical generator paraquat. pUM505 could provide *Pseudomonas* strains with a wide variety of adaptive traits such as virulence, heavy-metal and antibiotic resistance and oxidative stress tolerance which can be selective factors for the distribution and prevalence of this plasmid in diverse environments, including hospitals and heavy metal contaminated soils.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium widely distributed in the environment, including soil and water, as well as in association with various living host organisms (Battle et al., 2008). *P. aeruginosa* is one of the most prevalent causes of opportunistic infections in humans and is the most common cause of eventually fatal, persistent respiratory infections in cystic fibrosis patients (Battle et al., 2008). Furthermore, it has been shown that this bacterial species utilizes the same virulence determinants to infect different hosts, from plants to humans (Fajardo et al., 2008). Observations from a number of infection models indicate that the virulence of *P. aeruginosa* varies from strain to strain and that the genes encoding most of the characterized virulence determinants are

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located in the core genome and therefore are present in all strains. However, *P. aeruginosa* accessory genes contained on plasmids and genomic islands may contribute to the heterogeneity of virulence (Lee et al., 2006). pUM505 was first identified as a large conjugal plasmid in a clinical strain of *P. aeruginosa* conferring resistance to chromate and inorganic mercury (Cervantes-Vega et al., 1986; Cervantes and Ohtake, 1988). Resistance to chromate by pUM505 has been extensively studied, and functions by efflux of the chromate ion from the cytoplasm across the cytoplasmic membrane into the periplasm by the transmembrane protein ChrA (Cervantes et al., 1990; Alvarez et al., 1999). The resistance to mercury or other functions encoded by pUM505 have not been studied in detail. Therefore, in order to explore its genetic organization, we determined the complete nucleotide sequence of pUM505. In this paper, we present the detailed annotation of the nucleotide sequence of pUM505. By using computer-assisted DNA sequence analysis, we identified putative genetic determinants of pUM505 involved in virulence properties, replication, maintenance and those mediating heavy-metal resistance.

2. Materials and methods

2.1. Bacterial strains

Plasmid pUM505, originally obtained from *P. aeruginosa* strain PUM503 isolated from a hospital patient in Morelia, Michoacán, México (Cervantes-Vega et al., 1986), was transferred by conjugation to strain *P. aeruginosa* PU21 (FP^-, *ilv*, *leu*, Str^R, Rif^R), selecting for resistance to rifampicin and chromate and following the protocol described by Sambrook et al. (1989).

2.2. Susceptibility tests

Overnight cultures grown in nutrient broth (NB, Bioxon) were diluted 1:50 in tubes with 4 ml of fresh NB medium with varying amounts of either of the following compounds (from Merck Co. or Sigma Chemical Co.): mercuric chloride (0.5, 1.0, 2.0, 3.0, 4.0 μM), ciprofloxacin (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 μg/ml) or paraquat (1, 5, 10, 15, 20 μM). Cultures were incubated for 18–20 h at 37 °C with shaking, and growth was monitored as turbidity at 590 nm with a spectrophotometer.

2.3. DNA purification

Plasmid DNA was purified from overnight cultures of *P. aeruginosa* PU21(pUM505) grown in Luria Bertani broth (LB) (Bioxon) for 24 h at 37 °C, by means of the QIAGEN Large-Construct kit according to manufacturer's instructions. DNA was analyzed and quantified by agarose gel electrophoresis.

2.4. DNA sequencing

Purified pUM505 plasmid DNA (1.5 μg) was sequenced at the University of Arizona Genetics Core (Tucson, AZ, USA) on a Genome Sequencer FLX system (454 Life Sciences). Shotgun sequencing was performed according to the manufacturer's instructions using a Titanium SV emPCR Lib-L kit and one region of a four-region picotitre plate, resulting in 318, 311 reads with an average length of 271 bp. Sequences were assembled using GS De Novo Assembler version 2.0.01.12 (454 Life Sciences), generating 3978 contigs of at least 500 bp, most of which were low coverage (an average of 6 fold). BLAST analysis revealed that one contig with very high coverage (258 fold) contained the gene *chrA* already known to be part of the pUM505 plasmid (Cervantes et al., 1990). Connections between this contig and adjacent contigs with similarly high coverage were identified by looking for sequencing reads that were split between contigs by the assembly software. Continuing this process, a chain of 10 contigs, each having a minimum coverage of 200 fold, was assembled into a 123,322 bp sequence. Contig joins were checked by running a new assembly on all the reads from the 10 high-coverage contigs using GS De Novo Assembler version 2.3 with the option to limit reads to one contig.

2.5. DNA sequence analysis and annotation

After complete nucleotide sequencing of pUM505, potential open reading frames (ORFs) were searched by the RAST server (Rapid Annotation using Subsystem Technology) and using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI (http://www.ncbi.nlm.nih.gov/) prokaryotic database to confirm the results. Conserved domains were determinated by a search of Clusters of Orthologous Groups of proteins (COGs) in the NCBI data base. Molecular masses of encoded proteins were determined by ProtParam from Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/protparam.html). The complete sequence of pUM505 was searched for σ^{70}-dependent promoters using the Neural Network Promoter Prediction (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl) (Reese et al., 1996). Rho-independent bacterial terminators were searched using the program FindTerm (Softberry Inc.). Putative operons were determined by FGENESB: Bacterial Operon and Gene Prediction (http://www.linux1.softberry.com/berry.phtml?topic = fgenesb&group = programs&subgroup = gfindb). Insertion sequence elements within the pUM505 sequence were identified with IS FINDER (http://www-is.biotoul.fr/is.html). Search for genomic islands was made using CpG finger program from Softberry programs (http://www.linux1.softberry.com/berry.phtml). Global amino acid sequence similarities were calculated with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) from the European Bioinformatics Institute from the European Molecular Biology Laboratory. Proteins <100 aa were analyzed in order to determine whether they were truncated. The parameters used for this analysis were: a comparison with the length of homologous proteins, identification of insertion sequences or repeated inverted and analysis of arrangement of genes that encode these proteins. The annotated sequence of pUM505 is available in the NCBI database under the Accession No. HM560971.

3. Results and discussion

3.1. General features of plasmid pUM505

The complete nucleotide sequence of plasmid pUM505 could be assembled into a circular DNA sequence of 123,322 base pairs (bp), with an overall G + C content of 60.5%. pUM505 is 23 kb larger than the previous size estimation on the basis of agarose gel electrophoresis (Cervantes and Ohtake, 1988). Variation of G + C content throughout the replicon was evident, ranging from 58% to 65%. Fig. 1 presents a detailed genetic map of pUM505, indicating the approximate positions of genes and additional organizational features. The predicted coding regions of pUM505 showed a particular genetic organization, highlighting two well-defined regions corresponding to genomic islands, which correspond to 98 kb of total plasmid. The first one possessed a large island (~67 kb), which contained genes involved in virulence, plasmid replication, plasmid partitioning, plasmid maintenance, and conjugative transfer (Fig. 1). The second, smaller region (~31 kb) contained genes involved in heavy-metal resistance and mobility genes (Fig. 1).

A summary of the sequence data for pUM505, including length and molecular mass of predicted proteins, as well as best homologies to known proteins in databases, is shown in Table 1. The pUM505 sequence contained 138 complete coding regions, the majority of them encoded on the complementary DNA strand (75%), with respect to the predicted origin of replication (*oriV*) (Fig. 1). Most of the identified genes (46%) encode hypothetical proteins (H.P.). In addition, we identified mobile elements (10.9%), genes related to transfer functions (9.4%), heavy-metal resistance determinants (9.4%), metabolism-related genes (5.8%), and regulatory genes (3.6%), among others. The percentage of genes encoding H.P. was similar to the proportions reported in large plasmids such as pMOL28 at 171,459 bp (50%) and pMOL30 at 233,720 bp (40%) from *Cupriavidus metallidurans* CH34 (Monchy et al., 2007), or pWW0 at 116,580 bp from *Pseudomonas putida* (41%) (Greated et al., 2002). The proportion of genes encoding H.P. decreases in smaller plasmid such as pMccC7-H22 from *Escherichia coli* at 32,014 bp (36%) (Smajs et al., 2008), pDTG1 from *P. putida* NCIB 9816-4 at 83,042 bp (33%) (Dennis and Zylstra, 2004), and pB4 from *Pseudomonas* sp. at 79,370 bp (25%) (Tauch et al., 2003).

Most genes (80%) from pUM505 were located in potential operons; 30 putative operons of different sizes were identified: 25 contained 2–5 genes and five had 8–11 genes. Additionally, an automated search for σ^{70}-dependent promoters resulted in the prediction of a set of 106 putative promoter sequences, however, only 22 promoters

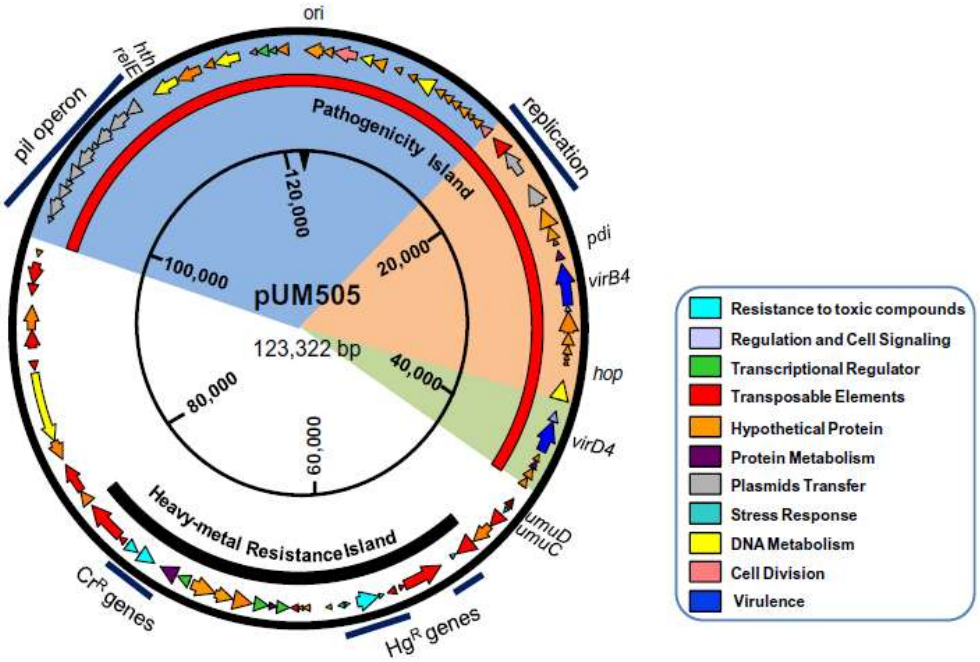

Fig. 1. Genetic map of *P. aeruginosa* plasmid pUM505. Deduced coding regions are shown by open arrows or arrowheads indicating the direction of transcription. The circular positions are indicated at intervals of 20,000 bp. The possible functions of the encoded proteins are according to the color code shown to the right. Two main regions of the plasmid are indicated: (1) pathogenicity island PAI (red bar); (2) heavy-metal resistance island (black bar). Mosaic-type structure, which consists of three blocks, is shown as different colored regions. Details are given in the text. The origin of replication (ori) is indicated. Putative operons containing coding regions predicted to be involved in conjugative transfer (*pil* operon), mercury resistance (Hg^R), and chromate resistance (Cr^R) are indicated by blue bars. Genes involved in replication, as well as other relevant genes (*umuD*, *umuC*, *virB4*, *virD4*) are indicated in the map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of location of predicted coding regions on plasmid pUM505.

Orf No.	Gene	pUM505 co-coordinates (5′–3′)	Length (bp)	No. of amino acids	Sequence identity to best homolog (Accession No.)[a]	PA14 locus tag
Orf1		1787–456	1332	443	H.P.[b]	PA14_59100[c]
Orf2		2539–1784	756	251	H.P.	PA14_59090
Orf3	*parB*	4300–2567	1734	577	92% to ParB from *P. aeruginosa* PA14 (ABJ13825)	PA14_59070
Orf4		4566–4297	270	89	H.P.	PA14_59060
Orf5	*damMTase*	5477–4587	891	296	96% to DNA adenine methyltransferase from *P. aeruginosa* PA7 (YP_001349802)	
Orf6		6496–5474	1023	340	H.P.	PA14_59050
Orf7		6729–6496	234	77	H.P.	PA14_59030
Orf8		6964–6722	243	80	H.P.	PA14_59020
Orf9		7214–6957	258	85	H.P.	PA14_59010
Orf10		7738–7211	528	175	H.P.	PA14_59000
Orf11		7876–8004	129	42	H.P.	
Orf12		8919–8299	621	206	H.P.	
Orf13	*dnaB*	10319–8976	1344	447	93% to DNA helicase from *P. aeruginosa* PA14 (YP_792897.1)	PA14_58990
Orf14		10534–10316	219	72	H.P.	PA14_58980
orf15		11246–10518	729	242	H.P.	PA14_58970
Orf16		11944–11243	702	233	H.P.	PA14_58960
Orf17		12630–11944	687	228	H.P.	PA14_58940
Orf18		13448–12627	822	273	H.P.	
Orf19		13951–13454	498	162	H.P.	PA14_58930
Orf20		14685–13948	738	245	H.P.	PA14_58920
Orf21	*parA*	15553–14687	867	288	98% to ParA protein from *P. aeruginosa* PA14 (ABJ13810)	PA14_58910
Orf22	*xerD*	17331–16030	1302	433	62% to XerD recombinase from *P. aeruginosa* PA14 (ABJ10138)	PA14_51650
Orf23	*traI*	19247–17328	1920	429	95% to TraI from pKLC102 (AAF22591)/CP102	PA14_60130
Orf24		19421–19720	297	99	93% to ParE-like domain from *P. aeruginosa* PA14 (YP_792993)	PA14_60050
Orf25		19859–20203	345	114	H.P.	PA14_60010
Orf26	*traG*	21782–20238	1545	514	92% to TraG from *P. aeruginosa* PA14 (ABJ13910)/	PA14_60020
Orf27		22126–21779	348	115	H.P.	PA14_60010
Orf28		23514–22126	1389	462	H.P.	PA14_60000
Orf29		24470–23532	939	312	H.P.	PA14_59990
Orf30		24901–24470	432	143	H.P.	PA14_59980
Orf31		25110–25328	219	72	H.P.	PA14_59970
Orf32	*pil*	25984–25325	660	219	95% to PilI from *P. aeruginosa* PA14 (ABJ13904)	PA14_59960
Orf33		26265–25981	285	94	H.P.	PA14_59950
Orf34	*virB4*	29204–26262	2943	980	97% to VirB4 from *P. aeruginosa* PA14 (ABJ13902)	PA14_59940
Orf35		29647–29204	444	147	95% to lipoprotein from *P. aeruginosa* PA14 (ABJ13901)	PA14_59930
ORf36		31130–29625	1506	501	H.P.	PA14_59920
Orf37		31998–31114	885	294	H.P.	PA14_59901
Orf38		32654–31995	660	219	H.P.	
Orf39		33037–32651	387	128	H.P.	PA14_59990
Orf40		33404–33048	357	118	H.P.	PA14_59980
Orf41		33661–33422	240	79	H.P.	PA14_59970
Orf42	*hop*	33996–33658	339	112	98% to Hop protein from *P. aeruginosa* PA14 (ABJ13894)	PA14_59860
Orf43		34395–34090	306	101	H.P.	PA14_59850
Orf44		35985–34504	1482	493	97% to DNA helicase from *P. aeruginosa* PA14 (ABJ13891)	PA14_59830
Orf45		37481–36735	747	247	97% to membrane protein from *P. aeruginosa* PA14 (ABJ13881)	PA14_59700
Orf46	*virD4*	39712–37481	2232	743	97% to VirD4 from *P. aeruginosa* PA14 (ABJ13880)	PA14_59690
Orf47		39985–39716	270	89	97% to dTDP-D-glucose 4,6-dehydratase from *P. aeruginosa* PA14 (ABJ13879) (truncated protein)	PA14_59680
Orf48		40494–39994	501	166	H.P.	PA14_59670
Orf49		41072–40491	582	193	98% to transglycosylase from *P. aeruginosa* C3719 (ABJ13877)	PA14_59660
Orf50		41812–41057	756	251	H.P.	PA14_59650
Orf51		42515–41823	693	230	H.P.	PA14_59640
Orf52		42733–43005	273	909	H.P.	
Orf53		43546–43403	144	47	H.P.	
Orf54		44281–43598	684	227	97% to Gifsy-2 prophage protein YedK (EAZ56186)	
Orf55	*umuD*	44431–44817	387	128	99% to UmuD from *P. aeruginosa* C7319 (EAZ56187)	
Orf56	*umuC*	44802–45017	215	72	77% to DNA polymerase V from *P. aeruginosa* C7319 (EAZ56188)	
Orf57	*xerC*	45042–46199	1158	385	20% to XerC from *E. coli* (AAC76814.1)	
Orf58		46210–47826	1617	537	H.P.	
Orf59		47807–49297	1491	496	99% to recombinase from *P. aeruginosa* PA7 (ABR82411)	
Orf60	*merR*	49750–49352	399	132	100% to MerR from pRA2 of *P. alcaligenes* (YP_025338)	
Orf61	*merT*	49825–50175	351	116	100% to MerT from pRA2 of *P. alcaligenes* (YP_025337)	
Orf62	*merP*	50188–50463	276	91	100% to MerP from pRA2 of *P. alcaligenes* (YP_025336)	
Orf63		50471–50683	213	70	H.P.	
Orf64	*tnpA*	53689–50696	2994	997	100% to TnpA from pRA2 of *P. alcaligenes* (YP_025335)	
Orf65		54103–53693	411	136	99% to protein of PIN superfamily (YP_001173906)	
Orf66		54342–54103	240	79	H.P.	
Orf67	*tniR*	54440–55054	615	204	100% to TniR from *Pseudomonas* sp. CT14 (YP_001966307)	
Orf68	*merE*	55343–55107	237	78	100% to MeE from *Pseudomonas* sp. CT14 (ABA26003)	
Orf69	*merD*	55705–55340	366	121	99% to MerD from *Pseudomonas* sp. CT14 (ABA26002)	
Orf70	*merA*	57368–55722	1647	547	100% to MerA from *Pseudomonas* sp. CT14 (ABA26001)	
Orf71	*merF*	57610–57365	246	81	100% to MerF from *Pseudomonas* sp. CT14 (ABA26000	
Orf72	*merP*	57888–57613	276	91	100% to MerP from *Pseudomonas* sp. CT14 (ABA25999)	
Orf73	*merT*	58254–57904	351	115	100% to MerT from *Pseudomonas* sp. CT14 (ABA25998)	
Orf74	*merR*	58326–58760	435	144	100% to MerR from *Pseudomonas* sp. CT14 (ABA26009)	
Orf75	*tnpA*	59264–58932	333	110	29% to Transposase TnpA (ABP81063) (truncated)	
Orf76		59270–59656	387	128	100% to Predicted nucleotidyltransferase (EER59501)	
Orf77		59672–59845	174	57	H.P.	
Orf78		60504–60830	327	108	H.P.	
Orf79		60827–61327	501	166	H.P.	
Orf80		61324–61695	372	123	H.P.	
Orf81	*tinR*	61689–62246	558	185	100% to TinR from *Burkholderia vietnamiensis* G4 (ABO60124.)	
Orf82		63248–62328	921	306	42% to LysR from *Thauera* sp. MZ1T (ACI02527)	
Orf83		63795–63268	528	175	53% to isochorismatase from *R. metallidurans* CH34 (ABF12518)	
Orf84		64901–63855	1047	348	32% to AraC from *P. fluorescens* Pf0–1 (ABA71950)	
Orf85		66443–65052	1392	463	56% to Protein of unknown function DUF1214 (ABP84480)	
Orf86		67851–66505	1347	400	H.P.	
Orf87		69674–67854	1821	606	H.P.	
Orf88		69928–70854	927	307	31% to AraC from *P. fluorescens* Pf-5 (AAY95613)	
Orf89		71008–72309	1302	433	52% to Pyrroloquinoline protein from *P. aeruginosa* (CAW28077)	
Orf90	*chrC*	72733–72473	261	86	54% to ChrC from pB4 plasmid (CAD24359) (truncated protein)	
Orf91	*chrA*	74043–72793	1251	416	100% to ChrA from *P. aeruginosa* (AAA88432)	
Orf92	*chrB*	75158–74214	945	313	88% to ChrB from pB4 plasmid (CAD24361)	
Orf93	*tnpR*	75426–75986	561	185	86% to TnpR from pB4 plasmid (NP_598137)	
Orf94	*tnpA*	75990–78956	2967	989	66% to TnpA from pB4 plasmid (NP_598138)	
Orf95		79240–80259	1020	339	H.P.	
Orf96		80256–82418	2163	720	89% to site-specific recombinase from *P. aeruginosa* PA7(ABR80879)	
Orf97		82698–82823	126	41	H.P.	
Orf98		84279–82855	1425	473	H.P.	
Orf99		89321–84279	5043	1680	39% to DNA and RNA helicase from H. chejuensis (ABC33653)	

(continued on next page)

Table 1 (continued)

Orf No.	Gene	pUM505 co-coordinates (5′–3′)	Length (bp)	No. of amino acids	Sequence identity to best homolog (Accession No.)[a]	PA14 locus tag
Orf100		90178–89504	675	224	74% to Transposase Tn*5* related protein (EAQ96150)	
Orf101		90663–90361	303	100	85% to Transposase IS*3*/IS*911* from *P. aeruginosa* PA7(ABR81266)	
Orf102	*umuC*	90853–91053	201	66	99% to UmuC (YP_001345482)	
Orf103		91100–92392	1293	429	44% to Integrase/recombinase from *Marinobacter* sp. (EBA00649)	
Orf104		92385–94121	1737	578	HLP.	
Orf105		95618–94815	804	267	99% to Transposase from *P. aeruginosa* PA7 (ABR85702)	
Orf106		97119–95611	1509	502	99% to Transposase from *P. aeruginosa* PA7 (YP_001345526)	
Orf107		97123–97248	126	41	HLP.	
Orf108		97425–97267	159	52	HLP.	
Orf109		98042–97563	480	159	HLP.	
Orf110		98501–98367	135	44	HLP.	
Orf111		98679–98503	177	58	HLP.	
Orf112		99143–98754	390	129	HLP.	PA14_59380
Orf113	*pilM*	100502–100065	438	145	93% to PilM from *P. aeruginosa* PA14 (AAP84202)	PA14_59360
Orf114	*pilV*	101899–100520	1380	459	88% to PilV from *P. aeruginosa* PA14 (ABJ13849)	PA14_59350
Orf115	*pilT*	102848–101892	957	318	85% to PilT from *P. aeruginosa* PA14 (ABJ13848)	PA14_59340
Orf116	*pilS*	103375–102845	531	176	98% to PilS from *P. aeruginosa* PA14 (ABJ13847)	PA14_59320
Orf117	*pilR*	104476–103397	1080	359	99% to PilR from *P. aeruginosa* PA14 (ABJ13846)	PA14_59310
Orf118	*pilQ*	106056–104476	1581	525	98% to PilQ from *P. aeruginosa* PA14 (ABJ13845)	PA14_59290
Orf119	*pilP*	106598–106065	534	177	97% to PilP from *P. aeruginosa* PA14 (ABJ13844)	PA14_59280
Orf120	*pilO*	107913–106588	1326	441	97% to PilO from *P. aeruginosa* PA14 (ABJ13843)	PA14_59270
Orf121	*pilN*	109626–107917	1710	569	99% to PilN from *P. aeruginosa* PA14 (ABJ13842)	PA14_59250
Orf122	*pilL*	110750–109626	1125	373	95% to PilL from *P. aeruginosa* PA14 (ABJ13841)	PA14_59240
Orf123	*relE*	110952–111251	300	99	55% to RelE from *K. pneumoniae* (ZP_06016753)	
Orf124	*hth*	111248–111538	291	96	92% to HTH from *P. alcaligenes* (AAD40346)	
Orf125		111953–111621	333	110	HLP.	
Orf126		112084–111950	135	44	HLP.	
Orf127		114070–112094	1977	658	97% to helicase from *P. aeruginosa* PA14 (ABJ13838)/	PA14_59210
Orf128		115956–114067	1890	629	HLP.	PA14_59200
Orf129		117028–116276	753	250	HLP.	
Orf130	*topA*	118956–117037	1920	639	97% to DNA topoisomerase I from *P. aeruginosa* PA14 (ABJ13835)	PA14_59180
Orf131		119269–119072	198	65	HLP.	PA14_59160
Orf132		119662119483–	180	59	HLP.	
Orf133	*ssb*	120256–119768	489	162	92% to SSB from *P. aeruginosa* PA14 (ABJ13832)	PA14_59150
Orf134		121116–120286	831	276	99% to regulatory protein Rha from *P. aeruginosa* PA7 (ABR81831)	
Orf135		121712–121164	549	182	95% to Integrase regulator R from *P. aeruginosa* PA14 (ABJ13831)	PA14_59140
Orf136		122587–121718	870	289	HLP.	PA14_59130
Orf137		122800–122603	198	65	HLP.	
Orf138		123286–122975	312	103	HLP.	

[a] Significant homology was determined by BLAST search and closest relationship of the deduced proteins is indicated.
[b] HLP. Hypothetical protein.
[c] locus tag of genes located on PAPI-1 from *P. aeruginosa* PA14.

Table 2
Putative promoters identified in pUM505 plasmid genes.

Coding sequence	pUM505 co-ordinates	Promoter sequence[a]	Direction[b]
ParA	**15759–15808**	GCTATTCCATA**TTGACA***TCACGTCAATGCGGGCC***TAATGT**TCGGCCCAGA	⇦
Hop	**34074–34123**	CTACCTGCTGT**TTTTCA***AGCGCGCAACCTCATAA***AAAAAT**ACCCCGAACC	⇦
Helicase	**34504–35985**	ACTCGCTAG**ATGAGA***ACCACGCGACAGCAAAAAG***CAGAAT**CAAAAAGATC	⇦
Gifsy	**44313–44362**	TCTGTTTATA**CAGTAGA***CGCTAACAGGATTTGCC***TACAAT**GCAGGTAGGC	⇦
Recombinase	**44945–44994**	TAAATGA**ATTACA***GTCCGCACCACCTTGA***TAGAAG**CCTGAGAAATTACAT	⇨
MerT	**49758–49807**	TCCTTGA**TTCCGT***ACTTTGGTACGGAGTT***TAGAAT**AGCACCTAGGCAATC	⇨
Transposase	**53960–54009**	GTTTCGAG**GTGGTGA***CCTCGCCGGATGCAACTCGA***TGAAAT**TCGTCGAGCC	⇦
MerT	**58299–58343**	CTCCAAA**TTGTTT***TCCATATCGCTTGACTCCG***TACATAA**CTACGGAAGTA	⇦
MerR	**58326–58760**	TACGT**TTGTCC***TTTCGAATCTGAATTGGA***TAGCTT**AAGCTTACTTCCGTA	⇨
TnpA transposase	**58932–59264**	ACTT**TTGATA***TTTCGTGCTGTCGCCTTCT***GAAAAT**GACAGGCGCGCGGCACG	⇦
Nucleotidyl transferase	**59270–59656**	GTTGGA**CTTGGGC***GATTTGGAAGAAGAAC***TGAAAT**CGCTGCTCGGCGTTG	⇨
H.P.	**69847–69896**	CACGACAGAG**TGATG***GAGCCGCATAACGCGCCTC***TATATC**GATGTCCGGG	⇦
AraC-type regulator	**69928–70854**	TGCAG**TTGCCG***CAGGCGGTGGCTGGCCAC***TACATT**CCGCTCCTGGCCGCG	⇨
ChrB	**75179–75228**	GTTGTGTAT**TAGATA***TAACTTATTGTAGTATTTG***TAACAT**TAATCATCTT	⇦
Transposase	**75426–75986**	CATT**TTGTGA***CACATGCCCGCGAAGGT***TATAGA**TTTCAGCCTGACAGAAA	⇨
Transposase	**90272–90321**	GCCG**TTGCTT***GAGGGTTTCTGCCAGTGGC***TATTAC**GCCTGGCAGGATCGT	⇦
H.P.	**84279–89321**	CCCTT**TTCAAA***CTGTCCATGGAAATGGGG***TAGAAC**CCAGACCTCCCGATC	⇦
H.P.	**97123–97248**	GACAG**CTTGTCG***CGGAAATACATCCGGCG***GATTTT**GCCCATCATTTCCAT	⇨
Periplasmic protein	**97563–98042**	CTGCTGACG**TTGTCG***GCAACCCTCCCCCAGT***AGTCAT**GATCTGAAAGACT	⇦
H.P.	**98754–99143**	CCTGC**TTGACC***TTGACACTTTCGCCCCCCGC***TTCGAT**GATGAGCGCGTGA	⇦
H.P.	**114067–115956**	CTCCT**TTTGAGG***TCAGTGGCCATGAAGGT***CATGAT**GTTCAGCTTCACCGT	⇦
Transcriptional regulator	**121164–121712**	GTTGGCCCATGG**TTGAAG***TCAAACAAACCGCCAC***TTTCAT**GGCATGGGAA	⇦

H.P. hypothetical protein.
[a] -35 and -10 regions indicated in bold are separated by a 17–19-bp spacer.
[b] The arrows indicate the orientation of each promoter relative to the map shown in Fig. 1.

were selected that showed a higher score and on the basis of their position relative to the ribosome binding site (Table 2). Other types of promoters such as sigma 32 or sigma 54 were not analyzed. Another feature of plasmid pUM505 is that its shows some duplicate genes; sequence analysis indicated that these are related genes and not identical sequences.

3.2. Pathogenicity island

The pUM505 sequence contained a region of ~67 kb (Fig. 1) that consisted of 78 ORFs (*orfs* 1–51 and 112–138) of which 64 (Table 1) have been found in pathogenicity islands PAPI-1 and PAPI-2 of *P. aeruginosa* PA14, a clinical isolate significantly more virulent in a wide range of hosts than the standard *P. aeruginosa* strain PAO1 (He et al., 2004). For this reason, we considered this region of pUM505 as a pathogenicity island (PAI). This island does not differ of the G + C content with respect to pUM505 plasmid (60.5%), however it possesses six genomic islets, whose G + C content is 64% and comprised approximately 50% of the total island. pUM505 PAI possesses genes involved in plasmid replication and conjugative transfer (Fig. 1), as well as genes encoding putative proteins with unknown functions, equivalent to ~56% of the genes of the genomic island.

Pathogenicity islands are typically large genomic regions (10–200 kb) that are present in genomes of pathogenic strains. The G + C content of PAIs commonly differs from the rest of the genome (Hacker and Kaper, 2000). PAIs were first described as chromosomal DNA regions, but increasing amount of sequence data from extrachromosomal elements supports the view that PAIs may also be part of plasmids or bacteriophage genomes (Hacker and Kaper, 2000). Virulence factors of pathogenic bacteria, such as adhesins, toxins, and protein secretion systems, may be encoded by PAIs (Hacker and Kaper, 2000). PAIs are acquired and exchanged by lateral gene transfer, and can be found in widely divergent species, making it difficult to ascribe their initial origins (He et al., 2004). The acquisition of genes in the form of PAIs distinguishes pathogenic isolates from nonpathogens (Lee et al., 2006). The *P. aeruginosa* accessory genome, which consists of bacteriophages, plasmids, and genomic islands found in some strains but not in others, may contribute to the heterogeneity of virulence (Battle et al., 2008).

The PAI of pUM505 displayed a mosaic-type structure consisting of three blocks of 40 (*orfs* 1–21 and 112–138), 18.4 (*orfs* 22–44) and 5.8 kb (*orfs* 45–51), shown as different colored regions in Fig. 1. These segments are related in sequence and genetic organization to gene clusters in the PAPI-1 island of *P. aeruginosa* PA14 (He et al., 2004). Mosaic-type structures consist of many DNA fragments which show a high similarity on the nucleotide level to chromosomal regions of other pathogenic strains or different virulence plasmids as well as sequence fragments without homology (Dobrindt et al., 2002). Mosaic-type structures are supported by studies of the organization of a number of well-characterized elements and closely-related elements (Mohd-Zain et al., 2004). PAIs similar to that of pUM505 have already been identified in islands of *Pseudomonas* strains 2192, PACS2, PA7, PA14 and C3719, which resemble a portion of PAIs associated with phage-like integrase genes (Würdemann and Tümmler, 2007), which belong to a large family of pKLC102-related GIs prevalent in beta- and gammaproteobacteria (Battle et al., 2008).

The blocks of pUM505 PAI presented synteny with the PAPI-1 island of *P. aeruginosa* PA14 (He et al., 2004) and pKLC102 of *P. aeruginosa* C (Battle et al., 2008). The syntenic region included *pil* genes involved in pilus biosynthe-

sis, and genes for DNA replication (40 kb block) (Fig. 1, blue block) (see below). The other syntenic region included virulence genes (18.4 kb block) (Fig. 1, orange block). The presence of a syntenic core in GIs suggests a fitness property of the conserved core genes acting collectively that has ensured their survival as a coherent syntenic whole (Mohd-Zain et al., 2004).

Identification of new pathogenicity islands is important because they likely encode novel virulence determinants that would increase our understanding of *P. aeruginosa* pathogenesis.

3.2.1. Genes with a putative role in virulence

A cluster of genes was identified in the PAI of pUM505. This cluster contains the *virD4*, *virB4* and *hop* genes, which have been associated with virulence. *virB4* and *virD4* genes (*orfs* 34 and 46, respectively) may encode two components typically associated with a multiprotein type IVA secretion (T4S) system corresponding to the secretion system related to type F and P plasmids (Guo et al., 2007; Juhas et al., 2008; Llosa et al., 2009). Further T4S genes were not found. The *hop* gene (*orf* 42) encoded a protein 98% similar to type III effector Hop protein from *P. aeruginosa* PA14 (YP_792974) (He et al., 2004). Hop proteins (denominated type III effectors), like the VirB/VirD4 secretion systems, have been related with virulence (Espinosa and Alfano, 2004; Jones and Dangl, 2006).

3.2.2. Transfer genes

A pilus biogenesis system was located as a cluster of *pil* genes (*orfs* 113–122) in the PAI of pUM505 (~10.5 kb) (Fig. 1; Table 1), forming a putative operon of 10 genes, *pilLNOPQRSUVM*. The *pil* operon of pUM505 contained genes for pilin protein PilS, prepilin peptidase PilU, outer membrane protein PilN, nucleotide-binding protein PilQ, integral membrane protein PilR, and pilus adhesin PilV (Kim and Komano, 1997). These data confirmed that pUM505 encodes conjugative sex pili. The pUM505 *pil* operon did not include a shufflon region that determines recipient specificity in liquid matings via generation of different adhesin types (Yoshida et al., 1999). This suggested that the genetic organization of the *pil* operon in pUM505 is appropriate for mating but lacks the option to evade the eukaryotic host immune response as it has evolved in enterobacteria. A difference in pUM505 was that *tra* plasmid transfer genes were not located close to the *pil* operon, as it occurs in the *pil* operon of *Salmonella enterica* serovar Typhi plasmid R64, an IncI conjugative plasmid (Juhas et al., 2008). Actually, *tra* genes are part of a cluster of genes involved in replication and stabilization of pUM505. The genes of the *pil* operon were similar in size, sequence, and gene arrangement to the *pil* operon of the PAPI-1 island from *P. aeruginosa* PA14 (Lee et al., 2006) but the pUM505 operon has an opposite direction of transcription, *pil* genes are encoding in 5′–3′ direction of the minus strand with respect to the coordinates from the *ori*. This orientation is essentially maintained throughout the pathogenicity island, suggesting that this region had a recombination event in plasmid pUM505. Another difference is that the *pil* operon of pUM505 contained a *pilU* gene in substitution of *pilT* (encoding PilT a putative lytic transglycosylase protein) in the *pil* operon of PAPI-1. *pil* operons with similarities to the one on pUM505 are located on genomic island pKLC102 of *P. aeuginosa* C and on the chromosome of *P. syringae* (Klockgether et al., 2004).

So, we conclude that *pil* genes from pUM505 might be involved in the formation of pili, and therefore in plasmid transfer, as well as in conferring virulence properties to *Pseudomonas* strains.

In addition to *pil* genes, pUM505 possesses *tra* genes. The *traI* gene (*orf* 23) encoded a putative relaxase 95% and 94% identical to TraI proteins from pKLC102 plasmid of *P. aeruginosa* and *P. aeruginosa* PA14, respectively (He et al., 2004).

The TraG protein from pUM505, encoded by the *traG* gene (*orf* 26), was 92% identical to its homologue from *P. aeruginosa* PA14 (He et al., 2004). TraG-like proteins are essential components of type IV secretion systems (Schröder and Lanka, 2003). However, an oriT was not identified.

3.2.3. Plasmid maintenance and replication genes

The putative origin of replication (*oriV*) of pUM505 spans about 800 bp and was located close to a gene cluster found on ~17 kb region that contained genes *parA*, *parB*, *xerD*, *xerC*, *traG and ssb*, that are probably involved in replication, recombination and segregation of the plasmid (Fig. 1; Table 1). The 3′ end of *oriV* region consisted of an A + T-rich region preceded by four palindromic sequences 15–27 bp in length, which may form loops. The core consisted of a 15-bp palindromic sequence GTTCGGCATCCGAAC (complementary sequence underlined). The 5′ end of the origin region displayed 16 highly-conserved 57-bp direct repeats, which consisted of a core represented by a G + C-rich stretch and a set of modules with similar but not identical sequence. The modules were 2–7 bp in length and appeared at the same position in all repeats. This type of *ori* is found in different GIs of *Pseudomonas* strains 2192, PACS2, PA7, PA14, C3719, and pKL2006C102 (He et al., 2004, Klockgether et al., 2004). These genomic islands share a similar organization whereby the 3′ end is more conserved than the 5′ end (Würdemann and Tümmler, 2007). However, the *oriV* region of pUM505 is located far (~14 kb) from the genes encoding the proteins for the replication machinery, different to other *ori* identified, which are usually located in the proximity to these types of genes.

The ParA protein from pUM505, encoded by the *par*A gene (*orf* 21), is 98% identical to ParA from *P. aeruginosa* PA14 (He et al., 2004). ParA is the prototype of a widespread and diverse superfamily of evolutionarily related proteins involved in a variety of functions, including plasmid and chromosome segregation, and cell division.

pUM505 also possesses a *parB* gene (*orf 24*) which encodes a protein which was 92% similar to ParB protein from *P. aeruginosa* PA14 (He et al., 2004). ParB is a DNA-binding protein that interacts directly with the plasmid partition sites that are normally composed of direct or inverted iterated sequences denominated *parS* (Bignell and Thomas, 2001). *par*B gene is located ~10 kb from the *par*A gene in pUM505; in contrast, in most plasmids these genes are grouped as an operon, so the functionality of these pair of genes could be questioned.

XerD protein from pUM505, encoded by *xerD* gene (*orf 22*) showed 62%/80% of identity/similarity to XerD from *P. aeruginosa* PA14 (He et al., 2004). XerD is a site-specific recombinase involved in stable plasmid inheritance (Sirois and Szatmari, 1995), and belongs to site-specific recombinases of the integrase family of proteins that catalyze recombination events via a phosphotyrosine intermediate (Reichmann and Hakenbeck, 2002). The functions of these enzymes include recombination events of plasmid DNA with the chromosome, maintenance of monomeric plasmid DNA, and regulatory DNA rearrangements (Reichmann and Hakenbeck, 2002). The XerC protein is encoded by *orf* 57 and shows 20%/61% of identity/similarity to the XerC protein (AAC76814.1) of *E. coli*. However, in pUM505 *xerD* and *xerC* genes are located very far (27.7 kb) with respect to each other, in contrast to *Streptococcus pneumoniae* and *Enterococcus faecalis* where both genes are arranged in a cluster (Reichmann and Hakenbeck, 2002). In addition, the identity between XerC and XerD from pUM505 is only 9%, a very low percentage compared with the same genes from *P. aeruginosa* PAO1 or *E. coli*. XerD/XerC might catalyze the integration of pUM505 in the chromosome, thus generating genomic islands.

Finally, the *ssb* gene (*orf 133*) encoded a protein 92% identical to the putative plasmid stabilization protein SSB from *P. aeruginosa* PA14 (He et al., 2004). The primary activity of SSB in DNA metabolism is to preferentially bind to ssDNA with high affinity independent of sequence, thereby stabilizing and controlling access to ssDNA, following separation of the double strands by helicases (Roy et al., 2009).

3.3. Genomic island with heavy-metal resistance genes

Genetic determinants involved in resistance to the heavy-metal mercury and the oxyanion chromate in pUM505 were grouped on a ~ 31-kb GI (*orfs* 59–94) (Fig. 1; Table 1). This region had an average G + C content of 64%, while the rest of the plasmid and the chromosome had an average G + C content of 61% and 66%, respectively. This small variation suggests that these genes have a different origin. Genes whose G + C content deviates from the mean G + C content of the organism are candidates for horizontal gene transfer (HGT) (Campbell et al., 1999). In addition, this region also contained several features frequently found to be associated with GIs, including the presence of flanking repeats, mobility genes (e.g. genes encoding integrases, resolvases, transposases), and IS elements (Fig. 2).

3.3.1. Mercury-resistance (Hg^R) determinants

The sequence of pUM505 plasmid contained two mercury-resistance (*mer*) putative operons (Barkay et al., 2003) (Fig. 1): a potential complete operon, constituted of *merRTPFADE* genes (*orfs* 68–74) (Fig. 2A), and an incomplete operon, formed only by *merRTP* genes (*orfs* 60–62) (Table 1). The complete *mer* operon of pUM505 was located between putative transposable elements and flanked by genes encoding a Bin resolvase (*orf* 67) and a putative type TnpA transposase (*orf* 75) (Fig. 2A). However, TnpA only possesses 110 aa, which suggests it to be a truncated protein which is probably not functional. In addition, an inverted repeat sequence characteristic of the Tn3 transposon was identified at the end of the *tnpA* gene (coordinates 59236). Another inverted repeat Tn3-type sequence was identified near the *Bin* gene (coordinates 54440), suggesting that this region is a remnant of an old transposition event (Fig. 2A). The sequence showed putative promoters upstream of the *merT* and *merR* genes of the *mer* operon (Fig. 2A). It may now be concluded that the mercury-resistance phenotype in pUM505 was conferred by the complete *mer* operon.

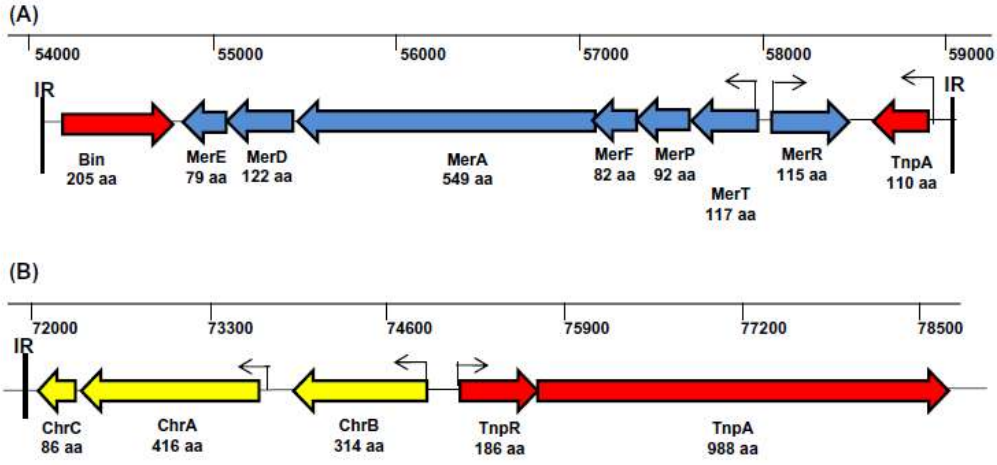

Fig. 2. Genetic organization of the putative heavy-metal resistance operons of pUM505. (A), mercury resistance determinant (*mer*) and (B), chromate resistance determinant (Cr^R). Coding regions are shown by open arrows indicating the direction of transcription. The size in amino acids (aa) of the encoded proteins are indicated. Putative mobile elements present in transposons are shown in red and the putative insertion sequence elements characteristic of Tn3-like transposons family are shown by black vertical lines (IR). Locations of putative promoters are indicated by small arrows. Details are given in the text.

The incomplete *mer* operon of pUM505 was adjacent to the complete *mer* operon, and was flanked by genes encoding a TnpA transposase (*orf* 64) and a recombinase (*orf* 59). The proteins encoded by these genes have 47, 46 and 49% of identity, respectively, with the proteins encoded by their counterparts located in the entire operon. The TnpA and Mer proteins encoded by pUM505 possess 100% of identity to their homologues from Tn*5563*, meanwhile the Tnp1 protein showed only 11% of identity to its homologue. Additionally, inverted sequences IS91 and a Tn*3*-type sequence were identified flanking this region (co ordinates 48090 and 55240, respectively). The incomplete *mer* operon of pUM505 is probably a remnant of rearrangements of transposition events, and may be not functional because it lacks the *mer*A gene for mercury reductase. A similar incomplete *merRTP* operon from pMOL30 plasmid of *C. metallidurans* was also reported as not functional (Monchy et al., 2007).

3.3.2. Chromate-resistance (Cr^R) determinants

The chromate-resistance determinant of pUM505 could be located on a putative *chrBAC* operon (*orfs* 90–92), situated within a potential transposon region that was flanked by genes encoding transposase/integrase TnpA (*orf* 94), and resolvase/integrase TnpR (*orf* 93) of a mobile element (Fig. 1; Table 1). The presence of a putative promoter upstream of *chrB* gene (Fig. 2B, Table 2) suggested that *chrBAC* operon can be transcribed starting from this region. However, the *chrA* gene may also be transcribed from its own promoter (Cervantes et al., 1990).

The *chrBAC* genes from pUM505 showed a similar organization to those of transposon Tn*5719* from plasmid pB4 from *Pseudomonas* sp. (Tauch et al., 2003) (Fig. 2B), the *chr* genes from *C. metallidurans* CH34 plasmid pMOL28 (Juhnke et al., 2002), the Tn*OtChr* transposon from *Ochrobactrum tritici 5bvl1* (Branco et al., 2008), plasmid 1 from *Shewanella* sp. ANA-3 (Aguilar-Barajas et al., 2008), and from other bacteria that possess uncharacterized homologues of the ChrA protein (Cervantes and Campos-García, 2007).

The *chrB* gene encoded a 315 aa protein with homology (88% identity) to the ChrB protein that has been proposed to play a regulatory role for expression of the ChrA transporter in *C. metallidurans* CH34 and *O. tritici* (Nies et al., 1990; Juhnke et al., 2002; Branco et al., 2008).

The *chrA* gene encoded the ChrA protein which was the first bacterial system described to confer chromate resistance (Cervantes et al., 1990; Alvarez et al., 1999). ChrA belongs to the CHR superfamily that includes hundreds of putative homologues from all three domains of life (Díaz-Pérez et al., 2007).

The *chrC* gene (previously named *orf 2*) encoded a chimeric protein of 86 aa (Cervantes et al., 1990) whose amino-terminal domain showed similarity to the first 41 amino acids of ChrC, a superoxide dismutase of 199 aa encoded on plasmid pMOL28 from *C. metallidurans* CH34 (Juhnke et al., 2002) and transposon Tn*OtChr* of *O. tritici* (Branco et al., 2008). The carboxyl-terminal domain of the truncated pUM505 ChrC showed similarity to *orf* 44 from *Tn*5719 transposon from pB4 plasmid of *Pseudomonas* sp. (Tauch et al., 2003). So, ChrC appeared to be a truncated protein which is probably not functional.

The presence of chromate resistance genes in pUM505 flanked by mobile elements suggested that they could have been acquired by HGT and might be a factor for the prevalence and widespread distribution of this plasmid in bacterial hosts.

Plasmid pUM505 could provide different *Pseudomonas* strains with a wide variety of adaptive traits such as virulence systems and heavy-metal resistance determinants, which could be selective factors for distribution of this plasmid in hospitals and other stressful habitats for the host. No studies on the prevalence of pUM505 have been conducted.

4. Other genes

Plasmid pUM505 contained several other genes that may encode proteins with important roles in prevalence of this plasmid in its bacterial hosts (Table 1). In general, these genes were located in clusters and some of them are briefly described below.

4.1. Toxin-antitoxin system

The cluster formed by *orfs* 123 and 124 encodes a toxin-antitoxin system. *Orf* 123 encodes a protein 55% identical to RelE protein (toxic component) of toxin-antitoxin system from *Klebsiella pneumoniae* (ZP_06016753).

Orf 124 encodes a putative transcriptional regulator 82% similar to the antitoxin HTH protein (Helix-turn-helix xenobiotics resistance family-like proteins) from *Pseudomonas alcaligenes* (YP_025327). Bacterial toxin-antitoxin systems (TAS) were characterized as plasmid-encoded molecular systems that ensure the persistence of a plasmid in host lineages during replication by making the cells "addicted" to the plasmid, so that only plasmid-containing daughter bacteria survive after cell division (Gerdes et al., 2005). This system could be an important mechanism to enhance the prevalence of pUM505 in *Pseudomonas* cells.

4.2. DNA adenine methyltransferase

Orf 5 encoded a DNA adenine methyltransferase (Dam-MTase) similar (96% identity) to a protein (YP_001349802) from *P. aeruginosa* PA7 (Roy et al., 2010) (Table 1). *Orf 5* was found in a cluster of 10 genes, eight of which encoded H.P. and one encoded the ParB protein afore mentioned. Dam-MTase from pUM505 was similar to proteins grouped in a D12 class N6 adenine-specific DNA methyltransferases (Kossykh and Lloyd, 2004). The presence of a gene encoding Dam-MTase in pUM505 may be related to enhancing plasmid segregational stability, although a gene encoding a restriction endonuclease (the second component of the restriction-methylation system) has not been identified.

4.3. DnaB helicase

Plasmid pUM505 contained a gene encoding a DnaB helicase (*orf* 13) located in a cluster of 10 genes (Fig. 1;

Table 1), one of them encoding the previously mentioned ParA protein (involved in segregation) and the other genes encoded H.P. DnaB is 93% identical to a DNA helicase from *P. aeruginosa* PA14 (He et al., 2004) that is a multifunctional enzyme with a number of distinct activities, including DNA binding, ATP hydrolysis, and DNA unwinding (Konieczny, 2003). DnaB forms a complex with DnaA (replication initiation factor) and DnaC (ATPase) (Konieczny, 2003), however pUM505 does not contain genes homologous to *dnaA* and *dnaC*.

4.4. UmuD and UmuC proteins

A possible operon was identified in pUM505 consisting of two genes (*orfs* 55 and 56) encoding a protein 99% identical to UV-light resistance protein A, and a small protein 77% identical to UV-light resistance protein B from *P. aeruginosa* C7319 (Mathee et al., 2008). The UV-light resistance protein A showed similarity with peptidase S24 from the LexA-like protein family. This family includes the lambda repressor CI/C2 family; LexA, the repressor of genes in the cellular SOS response to DNA damage; and the related UmuD proteins, which are lesion-bypass DNA polymerases induced in response to mutagenic DNA damage. UV-light resistance protein B showed a domain similar to Y-family of DNA polymerases, which includes members of the UmuC protein family (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?ascbin=8&maxaln=10&seltype=2&uid=119397). Based on these data, and considering that both genes of pUM505 are adjacent, we named them *umuD* and *umuC*. UmuD and UmuC of pUM505 may encode a system that confers additional protection to DNA damage in *P. aeruginosa*.

Another UmuC putative protein (*orf* 102) was detected, but this *orf* is flanked by genes encoding transposases and integrases and lacks a close *umu*D gene. The analysis of this region suggests that *orf* 102 was inserted into the pUM505 sequence and could be not functional.

5. Antibiotic resistance

Analysis of pUM505 did not identify any sequence encoding antibiotic resistance proteins reported to date. However, susceptibility tests of *P. aeruginosa* PU21 strain bearing pUM505 showed that it conferred resistance to the fluoroquinolone ciprofloxacin, an antibiotic that functions by interacting with type II topoisomerase (DNA gyrase and topoisomerase IV) (Luzzaro, 2008). *P. aeruginosa* PU21(pUM505) could grow at >0.8 μg/ml of ciprofloxacin, whereas the PU21 plasmidless strain was substantially inhibited by 0.4 μg/ml.

This finding suggests that pUM505 may possess a novel resistance system against fluoroquinolones.

6. Tolerance to oxidative stress

Sequence analysis of pUM505 identified *orf 32* encoding a putative protein 95% identical to a putative protein-disulfide isomerase (PDI) from *P. aeruginosa* PA14 (He et al., 2004). This gene is grouped in a cluster of 10 genes, which also includes the already mentioned *virB4* and *virD4* genes. PDIs catalyze formation of disulfide bonds in secretory proteins (Tian et al., 2008) and also have chaperone activity (Missiakas et al., 1994). PDI from pUM505 could be implied in formation of the correct pattern of disulfide bonds of proteins encoded by the plasmid. These activities of PDIs are probably related to protection of proteins from oxidative damage.

When transferred to *P. aeruginosa* PU21, pUM505 conferred moderately increased tolerance to the oxidative-stress generator paraquat. The presence of the plasmid allowed the exconjugant strain to grow at >10 μM paraquat, while the plasmidless strain was inhibited at 5 μM of the compound. These data suggest that mechanisms for resistance to oxidative stress may be encoded by pUM505, such as PDIs, and systems for DNA repair, such as the UmuD/UmuC proteins.

In summary, sequence analysis of pUM505 showed that this plasmid contains novel genes which may provide *Pseudomonas* strains with a wide variety of adaptive properties such as virulence, heavy-metal, antibiotic, and oxidative stress resistance systems, which can be involved in pathogenesis and evolution of bacterial hosts.

Acknowledgments

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References

Aguilar-Barajas, E., Paluscio, E., Cervantes, C., Rensing, C., 2008. Expression of chromate resistance genes from *Shewanella sp.* strain ANA-3 in *Escherichia coli*. FEMS Microbiol. Lett. 285, 97–100.

Alvarez, A.H., Moreno-Sánchez, R., Cervantes, C., 1999. Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. J. Bacteriol. 181, 7398–7400.

Barkay, T., Miller, S.M., Summers, A.O., 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. 27, 355–384.

Battle, S.E., Meyer, F., Rello, J., Kung, V.L., Hauser, A.R., 2008. Hybrid pathogenicity island PAGI-5 contributes to the highly virulent phenotype of a *Pseudomonas aeruginosa* isolate in mammals. J. Bacteriol. 190, 7130–7140.

Bignell, C., Thomas, C.M., 2001. The bacterial ParA–ParB partitioning proteins. J. Biotechnol. 13, 1–34.

Branco, R., Chung, A.P., Johnston, T., Gurel, V., Morais, P., Zhitkovich, A., 2008. The chromate-inducible *chrBACF* operon from the transposable element TnOtChr confers resistance to chromium(VI) and superoxide. J. Bacteriol. 190, 6996–7003.

Campbell, A., Mrázek, J., Karlin, S., 1999. Genome signature comparisons among prokaryote, plasmid, and mitochondrial DNA. Proc. Natl. Acad. Sci. USA 96, 9184–9189.

Cervantes, C., Campos-García, J., 2007. Reduction and efflux of chromate by bacteria. In: Nies, D.H., Silver, S. (Eds.), Molecular microbiology of heavy metals. Microbiology monographs, vol. 6. Springer–Verlag, Berlin, pp. 407–419.

Cervantes, C., Ohtake, H., 1988. Plasmid-determined resistance to chromate in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 56, 173–176.

Cervantes, C., Ohtake, H., Chu, L., Misra, T., Silver, S., 1990. Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. J. Bacteriol. 172, 287–291.

Cervantes-Vega, C., Chávez, J., Córdova, N.A., de la Mora, P., Velasco, J.A., 1986. Resistance to metals by *Pseudomonas aeruginosa* clinical isolates. Microbios 48, 159–163.

Dennis, J.J., Zylstra, G.J., 2004. Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain. J. Mol. Biol. 13, 753–768.

Díaz-Pérez, C., Cervantes, C., Campos-García, J., Julián-Sánchez, A., Riveros-Rosas, H., 2007. Phylogenetic analysis of the chromate ion transporter (CHR) superfamily. FEBS J. 274, 6215–6227.

Dobrindt, U., Blum-Oehler, G., Nagy, G., Schneider, G., Johann, A., Gottschalk, G.,

Espinosa, A., Alfano, J.R., 2004. Disabling surveillance: bacterial type III secretion system effectors that suppress innate immunity. Cell. Microbiol. 6, 1027–1040.

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J.C., Ghysels, B., Matthijs, S., Cornelis, P., Wiehlmann, L., Tümmler, B., Baquero, F., Martínez, J.L., 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS One 20, e1619.

Gerdes, K., Christensen, S.K., Lobner-Olesen, A., 2005. Prokaryotic toxin antitoxin stress response loci. Nat. Rev. Microbiol. 3, 371–382.

Greated, A., Lambertsen, L., Williams, P.A., Thomas, C.M., 2002. Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. Environ. Microbiol. 4, 856–871.

Guo, M., Jin, S., Sun, D., Hew, C.L., Pan, S.Q., 2007. Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type IV secretion apparatus. Proc. Natl. Acad. Sci. USA 104, 20019–20024.

Hacker, J., Kaper, J.B., 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. 54, 641–679.

He, J., Baldini, R.L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N.T., Lee, D., Urbach, J., Goodman, H.M., Rahme, L.G., 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. USA 101, 2530–2535.

Jones, J.D., Dangl, J.L., 2006. The plant immune system. Nature 444, 323–329.

Juhas, M., Derrick, W.C., Derek, W.H., 2008. Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell. Microbiol. 10, 2377–2386.

Juhnke, S., Peitzsch, N., Hubener, N., Grobe, C., Nies, D.H., 2002. New genes involved in chromate resistance in *Ralstonia metalliduran*s strain CH34. Arch. Microbiol. 179, 15–25.

Kim, S.R., Komano, T., 1997. The plasmid R64 thin pilus identified as a Type IV pilus. J. Bacteriol. 179, 3594–3603.

Klockgether, J., Reva, O., Larbig, K., Tummler, B., 2004. Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. J. Bacteriol. 186, 518–534.

Konieczny, I., 2003. Strategies for helicase recruitment and loading in bacteria. EMBO Rep. 4, 37–41.

Kossykh, V.G., Lloyd, R.S., 2004. A DNA adenine methyltransferase of *Escherichia coli* that is cell cycle regulated and essential for viability. J. Bacteriol. 186, 2061–2067.

Lee, D.G., Urbach, J.M., Wu, G., Liberati, N.T., Feinbaum, R.L., Miyata, S., Diggins, L.T., He, J., Saucier, M., Déziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L.G., Ausubel, F.M., 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol. 7, R90.

Llosa, M., Roy, C., Dehio, C., 2009. Bacterial type IV secretion systems in human disease. Mol. Microbiol. 73, 141–151.

Luzzaro, F., 2008. Fluoroquinolones and Gram-negative bacteria: antimicrobial activity and mechanisms of resistance. Infez. Med. 16, 5–11.

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J.M., Koehrsen, M., Rokas, A., Yandava, C.N., Engels, R., Zeng, E., Olavarietta, R., Doud, M., Smith, R.S., Montgomery, P., White, J.R., Godfrey, P.A., Kodira, C., Birren, B., Galagan, J.E., Lory, S., 2008. Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc. Natl. Acad. Sci. USA 105, 3100–3105.

Missiakas, D., Costa, C., Rainal, S., 1994. The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation. EMBO J. 13, 2013–2020.

Monchy, S., Benotmane, M.A., Janssen, P., Vallaeys, T., Taghavi, S., van der Lelie, D., Mergeay, M., 2007. Plasmids pMOL28 and pMOL30 of *Cupriavidus metalliduran*s are specialized in the maximal viable response to heavy metals. J. Bacteriol. 189, 7417–7425.

Mohd-Zain, Z., Turner, S.L., Cerdeno-Tarraga, A.M., Lilley, A.K., Inzana, T.J., Duncan, A.J., Harding, R.M., Hood, D.W., Peto, T.E., Crook, D.W., 2004. Transferable antibiotic resistance elements in *Haemophilus influenzae* share a common evolutionary origin with a diverse family of syntenic genomic islands. J. Bacteriol. 186, 8114–8122.

Nies, A., Nies, D.H., Silver, S., 1990. Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. J. Biol. Chem. 265, 5648–5653.

Reese, M.G., Harris, N.L., Eeckman, F.H. 1996. Large scale sequencing specific neural networks for promoter and splice site recognition. In: Hunter, L., Klein, T.E., (Eds.), Biocomputing (Proceedings of the 1996 Pacific Symposium). World Scientific Publishing, Singapore. http://www.fruitfly.org/seq-tools/promoter.pl.

Reichmann, P., Hakenbeck, R., 2002. A XerD recombinase with unusual active site motifs in *Streptococcus pneumoniae*. J. Mol. Microbiol. Biotechnol. 4, 101–110.

Roy, P.H., Tetu, S.G., Larouche, A., Elboume, L., Tremblay, S., Ren, Q., Dodson, R., Harkins, D., Shay, R., Watkins, K., Mahamoud, Y., Paulsen, I.T., 2010. Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. PLoS One 5, e8842.

Roy, R., Kozlov, A.G., Lohman, T.M., Ha, T., 2009. SSB protein diffusion on single-stranded DNA stimulates RecA filament formation. Nature 461, 1092–1097.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sirois, S., Szatmari, G., 1995. Detection of XerC and XerD recombinases in Gram-negative bacteria of the family Enterobacteriaceae. J. Bacteriol. 177, 4183–4186.

Smajs, D., Strouhal, M., Matejková, P., Cejková, D., Cursino, L., Chartone-Souza, E., Smarda, J., Nascimento, A.M., 2008. Complete sequence of low-copy-number plasmid MccC7-H22 of probiotic *Escherichia coli* H22 and the prevalence of mcc genes among human *E. coli*. Plasmid 59, 1–10.

Schröder, G., Lanka, E., 2003. TraG-like proteins of type IV secretion systems: functional dissection of the multiple activities of TraG (RP4) and TrwB (R388). J. Bacteriol. 185, 4371–4381.

Tauch, A., Schluter, A., Bischoff, N., Goesmann, A., Meyer, F., Puhler, A., 2003. The 79,370-bp conjugative plasmid pB4 consists of an IncP-1β backbone loaded with a chromate resistance transposon, the strA-strB streptomycin resistance gene pair, the oxacillinase gene blaNPS-1, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. Mol. Gen. Genomics 268, 570–584.

Tian, G., Kober, F.X., Lewandrowski, U., Sickmann, A., Lennarz, W.J., Schindelin, H., 2008. The catalytic activity of protein-disulfide isomerase requires a conformationally flexible molecule. J. Biol. Chem. 28, 33630–33640.

Würdemann, D., Tümmler, B., 2007. In silico comparison of pKLC102-like genomic islands of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 275, 244–249.

Yoshida, T., Kim, S.R., Komano, T., 1999. Twelve *pil* genes are required for biogenesis of the R64 thin pilus. J. Bacteriol. 181, 2038–2043.

CAPÍTULO II.

A plasmid-encoded UmuD homolog regulates expression of *Pseudomonas aeruginosa* SOS genes

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A plasmid-encoded UmuD homologue regulates expression of *Pseudomonas aeruginosa* SOS genes

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The *Pseudomonas aeruginosa* plasmid pUM505 contains the *umuDC* operon that encodes proteins similar to error-prone repair DNA polymerase V. The *umuC* gene appears to be truncated and its product is probably not functional. The *umuD* gene, renamed *umuDpR*, possesses an SOS box overlapped with a Sigma factor 70 type promoter; accordingly, transcriptional fusions revealed that the *umuDpR* gene promoter is activated by mitomycin C. The predicted sequence of the UmuDpR protein displays 23 % identity with the *Ps. aeruginosa* SOS-response LexA repressor. The *umuDpR* gene caused increased MMC sensitivity when transferred to the *Ps. aeruginosa* PAO1 strain. As expected, PAO1-derived knockout *lexA*$^-$ mutant PW6037 showed resistance to MMC; however, when the *umuDpR* gene was transferred to PW6037, MMC resistance level was reduced. These data suggested that UmuDpR represses the expression of SOS genes, as LexA does. To test whether UmuDpR exerts regulatory functions, expression of PAO1 SOS genes was evaluated by reverse transcription quantitative PCR assays in the *lexA*$^-$ mutant with or without the pUC_umuD recombinant plasmid. Expression of *lexA*, *imuA* and *recA* genes increased 3.4–5.3 times in the *lexA*$^-$ mutant, relative to transcription of the corresponding genes in the *lexA*$^+$ strain, but decreased significantly in the *lexA*$^-$/*umuDpR* transformant. These results confirmed that the UmuDpR protein is a repressor of *Ps. aeruginosa* SOS genes controlled by LexA. Electrophoretic mobility shift assays, however, did not show binding of UmuDpR to 5′ regions of SOS genes, suggesting an indirect mechanism of regulation.

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INTRODUCTION

pUM505 is a conjugative plasmid, originally isolated from a clinical *Pseudomonas aeruginosa* strain (Cervantes *et al.*, 1990), which confers resistance to chromate and inorganic mercury (reviewed by Ramírez-Díaz *et al.*, 2008). Sequence analysis revealed that pUM505 carries several putative adaptive genes, including *umuDC* genes, whose products are similar to the subunits of error-prone repair DNA polymerase V (Pol V), the *pdi* gene, associated with the protection of proteins from oxidative damage, and the *virB4*, *virD4* and *hop* genes, which are associated with virulence (Ramírez-Díaz *et al.*, 2011).

Expression of the chromosomal *Escherichia coli umuD* gene is upregulated as part of the SOS response to DNA damage (Nohmi, 2006). UmuD is initially produced as a 139 aa protein, which subsequently cleaves off its N-terminal 24 aa residues in a reaction dependent on a RecA/single-stranded DNA complex, giving rise to UmuD′ (Nohmi, 2006). The two forms of the *umuD* gene product play different roles in the cell. UmuD is implicated in a primitive DNA-damage checkpoint ($UmuD_2C$ complex) and prevents DNA polymerase IV-dependent −1 frameshift mutagenesis (Kivisaar, 2010), while the cleaved UmuD′ form facilitates UmuC-dependent mutagenesis via formation of DNA polymerase V after its association with the UmuC protein (the $UmuD'_2C$ complex) (Patel *et al.*, 2010).

Abbreviations: EMSA, electrophoretic mobility shift assay; MMC, mitomycin C; Pol V, DNA polymerase V; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR

Two supplementary tables and two supplementary figures are available with the online Supplementary Material.

A Plasmid-Encoded UmuD Homolog Regulates Expression of *Pseudomonas aeruginosa* SOS Genes

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Key words: LexA, pUM505 plasmid, SOS response

Abbreviations: MMC, mitomycin C; Pol V, DNA polymerase V

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Abstract

The *Pseudomonas aeruginosa* plasmid pUM505 contains the *umuDC* operon that encodes proteins similar to error-prone repair DNA polymerase V. The *umuC* gene appears to be truncated and its product is probably not functional. The *umuD* gene, renamed *umuDpR*, possesses an SOS box overlapped with a Sigma factor 70-type promoter; accordingly, transcriptional fusions revealed that the *umuDpR* gene promoter is activated by Mitomycin C (MMC). The predicted sequence of the UmuDpR protein displays 23% identity with the *P. aeruginosa* SOS-response LexA repressor. The *umuDpR* gene caused increased MMC sensitivity when transferred to the *P. aeruginosa* PAO1 strain. As expected, PAO1-derived knockout *lexA*-mutant PW6037 showed resistance to MMC; however, when the *umuDpR* gene was transferred to PW6037, MMC resistance level was reduced. These data suggested that UmuDpR represses the expression of SOS genes, as LexA does. To test whether UmuDpR exerts regulatory functions, expression of PAO1 SOS genes was evaluated by Reverse transcription-quantitative PCR (qRT-PCR) assays in the *lexA*$^{-}$ mutant with or without the pUC_umuD recombinant plasmid. Expression of *lexA*, *imuA*, and *recA* genes increased 3.4-5.3 times in the *lexA*$^{-}$ mutant, relative to transcription of the corresponding genes in the *lexA*$^{+}$ strain, but decreased significantly in the *lexA*$^{-}$/*umuDpR* transformant. These results confirmed that the UmuDpR protein is a repressor of *P. aeruginosa* SOS genes controlled by LexA. Electrophoretic mobility shift assays, however, did not show binding of UmuDpR to 5' regions of SOS genes, suggesting an indirect mechanism of regulation.

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However, the soil bacterium *Acinetobacter baylyi* strain ADP1 contains a chromosomal *umuDAb-umuC* operon that is unusual in content, regulation, and function as compared with the *E. coli* homolog (Hare *et al.,* 2006). The *umuDAb* gene encodes an extra 59-aa N-terminus region that is not encoded by other *umuD* genes, and the *umuC* gene is incomplete (Hare *et al.,* 2006; Fig. 1a). This operon

does not contain an SOS box, and its expression is constitutive (Hare *et al.,* 2006). UmuDAb carries out self-cleavage in a RecA-dependent manner after cells experience diverse forms of DNA damage, and shares features with both the Pol V component UmuD and the LexA repressor (Hare *et al.,* 2012). Recent work demonstrated that in the opportunistic pathogen *Acinetobacter baumannii* strain ATCC 17978, UmuDAb binds to and represses the promoters of *umuDC* homologs; thus, it might serve as a LexA analog for this genus (Aranda *et al.,* 2013). The significance of the regulatory role of UmuDAb is emphasized by the absence of a LexA homolog in *Acinetobacter* (Aranda *et al.,* 2013).

Because the majority of *P. aeruginosa* strains lack *umuDC* chromosomal genes, the *umuDC* operon from plasmid pUM505 may function as a regulator of the expression of genes related with the DNA damage response in this bacterium. The objective of this work was to characterize the function of pUM505 *umuDC* genes. We report here that the product of the pUM505 *umuD* gene is able to repress expression of SOS genes, and suggest that possession of this anti-SOS factor can be evolutionarily advantageous for the pUM505 plasmid.

Methods

Bacterial strains and culture conditions

P. aeruginosa PAO1 (from Dr. B. Iglewski's collection) (Li *et al.,* 2007) and *E. coli* W3110 (Hayashi *et al.,* 2006) are prototroph, standard strains that were used as hosts for recombinant plasmids. The PAO1-derived mutant strain PW6037 (*lexA*$^{-}$::ISphoA/bp) (obtained from the *P. aeruginosa* Mutant Library; Jacobs *et al.,* 2003)

was used for analysis of the expression of SOS genes. *E. coli* BL21-Codon-Plus(DE3)-RP (Stratagene) was used for protein overexpression.

Culture media employed were Nutrient broth (NB) or Luria–Bertani broth (LB; 1.5% agar for solid medium) (Green & Sambrook, 2012). If necessary, carbenicillin (400 µg ml^{-1}) or ampicillin (100 µg ml^{-1}) was added. Cells were routinely grown overnight at 37°C with shaking and the growth was monitored as Optical density (OD) at 600 nm (OD_{600}) with a spectrophotometer.

Genetic techniques and sequence analysis

General molecular genetic techniques were used according to standard protocols (Green & Sambrook, 2012). The cloning process of the constructs was verified by DNA sequencing carried out at the Department of Genetics, Cinvestav, Irapuato, Mexico. Protein sequence alignments were calculated with CLUSTALW algorithm and BioEdit Sequence Alignment Editor Software. Sequence similarities in protein and DNA databases were searched for using blastp and blastx programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Potential promoter sequences were searched for using the Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Transcription-factor binding sites were determined using Virtual Footprint and PRODORIC (http://www.prodoric.de/vfp/vfp_promoter.php).

RT-PCR assays

Cells were grown at an OD_{600} of 0.6 (mid-exponential phase) in NB at 37°C with shaking. Total RNA was isolated by using the Tri reagent (Molecular Research

120 Center, Inc.). DNA was removed with RQ1 RNase-free DNase (Promega), and
121 RNA was quantified by spectrophotometric analysis at 260 nm. Reverse
122 transcription (RT)-PCR was performed with 50 ng of RNA samples and the one-
123 step Access RT-PCR System kit (Promega). The oligonucleotides used, umuD_F
124 and umuD_R (Table S1), amplify a 222 bp fragment containing the overlap region
125 of the *umuDpR* and *umuC* genes (Fig. 1a). Positive and negative controls were
126 performed with PCR master mix (Promega) and the pairs of primers described in
127 Table S1 using plasmid DNA or total RNA as templates, respectively.
128
129 **Transcriptional fusions**
130 A 378-bp DNA fragment containing the putative *umuDpR* gene 5´ regulatory region
131 was amplified by PCR from purified pUM505 plasmid DNA employing
132 oligonucleotides listed in Table S1. Primers were located 243 bp upstream and 135
133 bp downstream of the *umuDpR* start codon, respectively. PCR fragments were
134 purified and cloned into the pJET1.2/blunt vector (Fermentas). Recombinant
135 plasmids were transferred by electroporation into *E. coli* strain W3110, and
136 transformants were selected on LB agar plates with ampicillin. DNA fragments from
137 recombinant plasmids were obtained by digestion with *Eco*RI and *Bam*HI
138 endonucleases and subcloned into the corresponding sites of the pLP170 vector,
139 which possesses a promoterless *lacZ* reporter gene (Preston *et al.,* 1997).
140 Recombinant plasmid pLP_umuDC was then transferred by electroporation into the
141 *P. aeruginosa* strain PAO1 and transformants were selected on LB agar plates with
142 carbenicillin. β-galactosidase activities were determined utilizing the chromogenic
143 substrate ONPG (Sigma) in permeabilized cells, as described previously (Acosta-

144 Navarrete *et al.,* 2014). Enzyme activities were corrected by subtracting the values
145 obtained from cultures of cells bearing the pLP170 vector without insert.
146
147 **Cloning of the *umuDpR-C* genes**
148 The *umuDpR-C* genes, or the individual *umuDpR* gene, were obtained by PCR
149 from pUM505 plasmid DNA using specific primers (Table S1). PCR fragments
150 were purified, cloned into the pJET1.2/blunt vector, and transferred into the *E. coli*
151 strain W3110, as described in the previous section. DNA fragments were obtained
152 by digestion with *Bam*HI and *Hin*dIII and subcloned into the corresponding sites of
153 the pUCP20 binary vector (West *et al.,* 1994). Recombinant plasmids pUC_umuDC
154 or pUC_umuD were transferred into the *P. aeruginosa* strain PAO1, as described
155 in the previous section.
156 The *umuDpR* gene coding region was cloned into the pJET1.2/blunt vector as
157 described in the previous paragraph. DNA fragments were obtained by digestion
158 with *Bam*HI and *Eco*RI and subcloned into the corresponding sites of the
159 pTrcHis2A expression vector (Invitrogen), which provides a 6-His tag at the C-
160 terminal end of the cloned product. Recombinant plasmid pTrcUmuD-His, encoding
161 tagged UmuDpR-His protein of 174 amino acid residues (19.2 kDa), was
162 transferred into *E. coli* BL21-Codon-Plus(DE3)-RP, selecting transformants on LB
163 agar plates with ampicillin.
164
165 **Susceptibility tests**

Bacteria were grown by diluting 1:100 overnight cultures in tubes with 4 ml of NB with increasing amounts of Mitomycin C (MMC) (Sigma). Cultures were incubated for 18 h at 37°C with shaking and growth was measured as OD_{600}.

cDNA synthesis and quantitative PCR (qPCR). For expression measurements, total RNA was isolated and quantified as described in the section of RT-PCR. cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega) and random hexamers according to the manufacturer´s instructions. The expression analysis of SOS genes was performed by qPCR using the comparative Ct method (ΔΔCt) with a StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out with a USB VeriQuest SYBR Green qPCR Master Mix according to the manufacturer's instructions. qPCR primers (Table S1) were designed using Biosearch Technologies software (https://www.biosearchtech.com/support/applications/realtimedesign-software). Relative expression of SOS genes was normalized with expression values obtained from the *rpoB* gene, which encodes the RNA polymerase β subunit (Qi *et al.,* 2001). Specificity of PCR assays was determined from dissociation curves generated after the qPCR reactions. Appropriate positive and non-template controls were included in every test run. The qPCR data were obtained from three independent experiments performed in duplicate. The results are reported as the means ± standard error (SE).

Electrophoretic mobility shift assays (EMSA)

189 Overnight cultures of *E. coli* BL21-Codon-Plus(DE3)-RP (pTrcUmuD-His) were
190 diluted 1:100 in 100 ml of LB containing 0.1 mM IPTG and incubated for 18 h at
191 37°C with shaking. Protein purification was performed essentially as described
192 previously (Watanabe & Takada, 2004). Cells were disrupted by sonic oscillation to
193 clarity and debris were removed by centrifugation. The supernatant was loaded
194 onto a Nickel–NTA resin (Qiagen) packed into a column; UmuDpR-His (19 kDa)
195 was recovered by elution with 250 mM imidazole and dialyzed as described
196 previously (Aranda *et al.,* 2008). Purification was monitored by 14 % SDS-PAGE.
197 For EMSA, DNA fragments of about 400 bp containing the 5´ regions of each of the
198 SOS genes were amplified by PCR using specific primers (Table S1). Purified DNA
199 fragments (100 ng) and varied amounts of purified UmuDpR-His protein were
200 mixed in binding buffer [10 mM Tris-HCl (pH 8.0), 10 mM HEPES, 50 mM KCl, 1
201 mM EDTA, 5% glycerol, 0.5 mM DTT and 0.1 mg ml^{-1} BSA] and incubated for 30
202 min at room temperature. Binding reaction mixtures were loaded onto 6% native
203 PAGE, separated at 100 V for 80 min in TAE buffer (Green & Sambrook, 2012)
204 and stained with 0.01% ethidium bromide for 30 seg. The *P. aeruginosa* PAO1
205 LiuR transcriptional regulator (ORF PA2016), and a DNA fragment containing *liuR*
206 gene 5' regulatory region, a gift of Dr. J. Campos-Garcia, were used as positive
207 control of the gel retardation assays.
208
209 **Results and discussion**
210 **The *umuDC* genes of the pUM505 plasmid**
211 Due to annotation errors in the nucleotide sequence of the pUM505 *umuD* (named
212 as *umuDpR*, see later), and in the *umuC* reading frames previously reported

(Ramírez-Díaz *et al.,* 2011), we identified new start codons for each gene, which are now predicted to encode proteins of 143 aa and 46 aa, respectively (Fig. 1a). UmuDpR of pUM505 exhibited sequence identities of 43% to *E. coli* K12 UmuD, the best studied UmuD protein, a component of DNA polymerase Pol V (Kitagawa *et al.,* 1985) (Fig. 1a), and of 41% to *A. baylyi* ADP1 UmuDAb; the *A. baylyi* UmuDAb protein is unusual because it possesses an extra 59-aa N-terminal region (Hare *et al.,* 2006) (Fig. 1a) and has been shown to function as a transcriptional regulator (Hare *et al.,* 2012).

UmuDpR is more similar to the *E. coli* UmuD protein and its homologs RumA and MucA (35–43% amino acid identity) than to the LexA repressor (23 and 22% of identity to the *P. aeruginosa* PAO1 and *E. coli* K12 homologs, respectively) (Fig. 1b). Moreover, no similarity exists between the DNA-binding N-terminal domain of LexA and the same region of UmuDpR (Fig. 1b). However, UmuDpR possesses conserved residues (Fig. 1b) that are required for RecA-facilitated self-cleavage in UmuD (the Pol V subunit) (Sutton *et al.,* 2001), the LexA repressor (Luo *et al.,* 2001), and the UmuDAb regulator (Hare *et al.,* 2006; 2012). The mechanism by which these proteins are cleaved is similar in that the active site contains a Ser-Lys dyad and the cleavage site is the dipeptide sequence (Ala/Cys)-Gly (Hare *et al.,* 2006) (Fig. 1b). However, a two-amino-acid motif (Leu-Arg) required for efficient UmuD self-cleavage in *E. coli* (Sutton *et al.,* 2001) is not present in UmuDpR (Fig. 1b); this region is more similar to the corresponding sequences of the LexA and UmuDAb transcriptional regulators. These data suggested that UmuDpR may also function as a transcriptional regulator; for this reason, the pUM505 *umuD* gene was herein renamed *umuDpR* (for *umuD p*lasmid *R*egulator).

The pUM505 *umuC* gene encodes a putative protein that is much smaller than its homologs with established function, such as *E. coli* UmuC (Kitagawa *et al.,* 1985) (Fig. 1a). pUM505 UmuC presents sequence identities of 38% to the N-terminal 63 aa of *E. coli* UmuC and of 44% to *A. baylyi* UmuC*, another small polypeptide that is considered a truncated protein (Hare *et al.,* 2006) (Fig. 1a). Thus, the *umuC* gene of pUM505 also appears to encode a truncated product that will be referred to as UmuC*, that is probably not functional (see later).

umuDpR-umuC* genes constitute an operon

In the majority of bacteria, *umuDC* genes, as well as their homologs, are usually adjacent, their coding regions overlap, and they form operons (Ippoliti *et al.,* 2012). The reading frames of *umuDpR-umuC** genes of pUM505 overlap by 14 nucleotides, suggesting that they form an operon (Fig. 1a). Furthermore, *in silico* analysis of the 5´ regions of the *umuDpR* and *umuC** genes identified a potential promoter only for *umuDpR* (Fig. 1a). To confirm the transcriptional linkage of *umuDpR* and *umuC** genes, RT-PCR analysis was performed. Primers were designed to amplify cDNA synthesized from a transcript spanning the reading frame overlap of *umuDpR-umuC** genes (Fig. 1a). When total RNA from *P. aeruginosa* PAO1 (pUM505) was analyzed by RT-PCR, a faint but consistent ~200-bp band was detected (Fig. 2a, lane 2). The corresponding DNA fragment was also detected in positive, but not in negative controls (Fig. 2a, lanes 3 and 4, respectively). These data demonstrate that the *umuDpR* and *umuC** genes from pUM505 plasmid are cotranscribed into a bicistronic messenger RNA, and suggest

that they form an operon whose expression depends on the *umuDpR* putative promoter.

Induction of the *umuDpR* gene by Mitomycin C

The putative regulatory region of the *umuDpR* gene presents a potential promoter with consensus –35 and –10 boxes related with general Sigma factor 70-type promoter sequences (Fig. 2b). Sequence analysis of the 5´ region of the *umuDpR* gene identified a putative transcription-factor binding region with similarity to an SOS box, which partially overlaps with the –10 box of the promoter sequence (Fig. 2b). The SOS box of *umuDpR* exhibits a perfect match with regulatory regions of the *umuDC* genes of *E. coli* (Kitagawa *et al.,* 1985) and of *rulAB* homologs from *Pseudomonas putida* plasmid pWW0 (Tark *et al.,* 2005). Expression of these genes in *E. coli* and *P. putida* responds to DNA damage (Kitagawa *et al.,* 1985; Tark *et al.*, 2005), suggesting that *umuDpR-umuC** genes from pUM505 may also respond likewise. Supporting this hypothesis, when total RNA from *P. aeruginosa* PAO1 (pUM505) cells exposed to the DNA damage-inducing agent MMC was analyzed by RT-PCR, an intense band, which contains the overlap region of *umuDpR-umuC**, was detected (Fig. 2a, lane 1). To confirm this result, the *umuDpR* putative regulatory region was cloned upstream of the promoterless *lacZ* reporter gene in pLP170 vector and β-galactosidase activity was measured. NB-grown *P. aeruginosa* PAO1 cells carrying plasmid pLP_umuDC showed significant LacZ activity (Fig. 2c, empty bars), indicating that the *umuDpR* promoter is functional under non-stressing conditions. This result is in agreement with a previous report showing that pseudomonads have higher baseline expression of

particular SOS regulon genes, such as *umuDC* genes, even in the absence of DNA damage (Kivisaar, 2010). However, when cells were exposed to subinhibitory levels of MMC, higher β-galactosidase activity was observed (Fig. 2c, black bars); *umuDpR* promoter increased the activity about 2.5 times respect to the untreated control after 3-h incubation. It can be concluded from these data that expression of the *umuDpR-umuC** operon is induced by DNA damage caused by MMC.

Functional analysis of *umuDpR-umuC genes**

As mentioned previously, the UmuDpR protein exhibits identity with the LexA repressors of *P. aeruginosa* and *E. coli*, as well as with the UmuDAb regulators of *A. baumannii* and *A. baylyi*. Furthermore, UmuDpR possesses residues required for self-cleavage that more closely resemble those of the LexA-type repressors than those from the UmuD proteins that constitute the Pol V enzyme. To elucidate the function of UmuDpR as a regulator of the SOS response, the *umuDpR-umuC** genes were amplified by PCR and cloned into the high-copy-number pUCP20 vector. The resulting recombinant plasmid, pUC_umuDC, was transferred into *P. aeruginosa* PAO1 and susceptibility tests to MMC were performed. *P. aeruginosa* PAO1 transformants expressing *umuDpR-umuC** genes showed increased sensitivity to MMC as compared with the control PAO1 (pUCP20) strain (Fig. 3a). This behavior may be attributed to overproduction of UmuDpR, which represses expression of SOS genes thus diminishing the DNA damage repair effects of the SOS pathway. The small size of pUM505 UmuC* and the fact that it lacks domains involved in DNA and protein interaction, suggested that is not functional. Accordingly, *P. aeruginosa* PAO1 transformants expressing the *umuDpR* gene

alone showed similar growth inhibition by MMC on comparison with the strain with the *umuDpR-umuC** gene pair (Fig. 3a). This result confirms that UmuDpR is responsible for the higher MMC susceptibility observed. The sequence of the *umuDpRC** operon appears to have been corrupted by mutation and, although its expression continues to respond to DNA damage, the truncated UmuC* protein is not functional.

To examine whether the UmuDpR protein is able to complement the function of the LexA repressor, the pUC_umuD plasmid was transferred into strain PW6037, a *P. aeruginosa* PAO1-derived knockout mutant affected in the *lexA* gene. The identity of the *lexA*$^{-}$ mutant was confirmed by PCR (data not shown), employing specific primers (Table S1). As expected, the PW6037 strain was more resistant to MMC than the wild-type PAO1 strain (Fig. 3b). This result is in agreement with previous reports showing that *E. coli* mutants affected in the *lexA* gene are more resistant to DNA damaging agents than wild-type strains, because several genes, whose products participate in DNA repair, are constitutively expressed (Walker, 1984; Fernández de Henestrosa *et al.,* 2000). However, when the PW6037 strain was transformed with the pUC_umuD plasmid, it displayed an intermediate susceptibility phenotype to MMC (Fig. 3b); this result indicates that UmuDpR is able to complement, at least partially, the function of the *P. aeruginosa* LexA protein and suggests that UmuDpR could act as a repressor of SOS response genes controlled by LexA.

UmuDpR regulates expression of SOS genes

Because the *P. aeruginosa* PAO1 genome lacks *umuDC* genes (Kivisaar, 2010), and the UmuDpR protein possesses characteristics of LexA-type repressors, we tested directly whether UmuDpR is able to regulate the expression of *P. aeruginosa* SOS genes. For this purpose, we selected *phl*, *imuA*, *lexA,*and *recA* genes (Table S2), which are part of the SOS regulon in other bacteria; all these genes are regulated by LexA in the PAO1 strain (Cirz *et al.,* 2006).

qRT-PCR analyses were carried out using total RNA from wild-type *P. aeruginosa* PAO1 strain, the PW6037 *lexA*$^{-}$ mutant, and PW6037 mutant transformed with the pUC_umuD plasmid. The results showed that expression of *recA, imuA,* and *lexA,* genes was upregulated 5.3-, 4.3-, and 3.4-fold, respectively, in the *lexA*$^{-}$ mutant compared to expression of the correspondent genes in the wild-type *lexA*$^{+}$ strain (Fig. 4). This behavior was expected, given the repressor nature of the LexA protein. Interestingly, the presence of *umuDpR* in the *lexA*$^{-}$ mutant caused the expression of these three genes to decrease significantly, although to different levels (Fig. 4). These data confirmed that UmuDpR is a transcriptional regulator able to repress SOS genes expression. This transcriptional control included the genes encoding RecA and LexA, the main modulators of the SOS response, and the *imuA* gene, which has been involved in DNA damage-induced mutagenesis in the α-Proteobacterium *Caulobacter crescentus* (Galhardo *et al.,* 2005).

Transcription of the *phl* gene, which encodes a photolyase-like enzyme that repairs UV-induced DNA lesions, showed no significant changes in both the *lexA*- mutant and in its *umuDpR* transformant (Fig. 4). Regulation of SOS genes by UmuDpR probably varies due to differences in its affinity to the regulatory regions of the distinct genes. To further analyze the role of the UmuDpR protein as a

355 transcriptional regulator, interaction of UmuDpR protein with SOS genes operators
356 was tested. The *umuDpR* gene was first cloned into an expression vector adding a
357 6-His tag at the protein C-terminus and the UmuDpR-His recombinant protein was
358 overexpressed (Figure S1). UmuDpR-His was recovered at high purity after nickel
359 affinity chromatography (Fig. S1) and it was used in EMSA. These assays,
360 however, did not show any specific binding of UmuDpR to the 5' regions of SOS
361 genes (Fig. S2 and data not shown). The *P. aeruginosa* LiuR transcriptional factor
362 displayed a clear retardation effect (Fig. S2), thus validating the EMSA. These
363 data, and the fact that UmuDpR lacks the DNA-binding N-terminal domain of LexA
364 (Fig 1b), suggest that transcriptional regulation by UmuDpR involves an indirect
365 mechanism. Thus, UmuDpR may require an additional cofactor(s) to accomplish its
366 regulatory functions, as previously postulated for the homologous UmuDAb protein
367 encoded in the *Acinetobacter baylyi* chromosome (Hare *et al*., 2012).
368 Inhibition of SOS response induction by plasmid-encoded gene products was first
369 reported in *E. coli* by Bagdasarian *et al.* (1980, 1986); these authors identified the
370 PsiB protein from the *E. coli* conjugative plasmid R100.1 as responsible of
371 inhibition of the generation of an SOS signal. It was later shown that PsiB does not
372 affect expression of the SOS pathway (Bagdasarian *et al.,* 1992), but directly binds
373 the RecA protein (Petrova *et al.,* 2009), thus impairing its DNA-damage repair
374 activities. To our knowledge, the UmuDpR protein represents the first report of a
375 LexA-type transcriptional regulator that is encoded by a plasmid. As previously
376 postulated by Bagdasarian *et al.* (1986, 1992) for the PsiB protein, UmuDpR may
377 protect the plasmid conjugative transfer process, which involves the transient
378 formation of single-stranded DNA, which may trigger the potentially deleterious

SOS response. Thus, the SOS response not only involves activities to survive or to change, but also includes actions devoted to sharing information with neighboring cells (Baharoglu & Mazel, 2014). Possession of an SOS response repressor could be evolutionarily advantageous for the conjugative pUM505 plasmid.

In summary, our results indicate that UmuDpR from pUM505 participates in regulation of the expression of SOS genes by an indirect mechanism, and suggest that the functioning of this protein as an anti-SOS effector can be an evolutionary strategy to maintain the integrity of the plasmid and to adapt it to new bacterial hosts.

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References

Acosta-Navarrete, Y. M., León-Márquez, Y. L., Salinas-Herrera, K., Jácome-Galarza, I. E., Meza-Carmen, V., Ramírez-Díaz, M. I. & Cervantes, C. (2014). Expression of the six chromate ion transporter homologues of *Burkholderia xenovorans* LB400. *Microbiology* **160**, 287-295.

Aranda, J., Garrido, M.E., Cortés, P., Llagostera, M. & Barbé, J. (2008). Analysis of the protective capacity of three *Streptococcus suis* proteins induced under divalent-cation-limited conditions. *Infect Immun* **76**, 1590-1598.

Aranda, J., Poza, M., Shingu-Vázquez, M., Cortés, P., Boyce, J.D., Adler, B., Barbé, J. & Bou, G. (2013). Identification of a DNA-damage-inducible regulon in *Acinetobacter baumannii*. *J Bacteriol* **195**, 5577-5582.

Bagdasarian, M., D'Ari, R., Filipowicz, W. & George, J. (1980). Suppression of SOS functions in an *Escherichia coli tif-1* mutant by plasmid R100.1. *J Bacteriol* **141**, 464-469.

Bagdasarian, M., Bailone, A., Bagdasarian, M.M., Manning, P.A., Lurz, R., Timmis, K.N. & Devoret, R. (1986). An inhibitor of SOS induction specified by a plasmid locus in *Escherichia coli*. *Proc Natl Acad Sci USA* **83**, 5723-5726.

Bagdasarian, M., Bailone, A., Angulo, J.F., Scholz, P., Bagdasarian, M.M. & Devoret, R. (1992). PsiB, and anti-SOS protein, is transiently expressed by the F sex factor during its transmission to an *Escherichia coli* K-12 recipient. *Mol Microbiol* **6**, 885-893.

Baharoglu, Z. & Mazel, D. (2014). SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiol Rev* **38**, 1126-1145.

Cervantes, C., Ohtake, H., Chu, L., Misra, T.K. & Silver, S. (1990). Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. *J Bacteriol* **172**, 287-291.

Cirz, R.T., O'Neill, B.M., Hammond, J.A., Head, S.R. & Romesberg, F.E. (2006). Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* **188**, 7101-7110.

Fernández de Henestrosa, A.R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H. & Woodgate, R. (2000). Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. *Mol Microbiol* **35**, 1560-1572.

Fernández de Henestrosa, A.R., Cuñé, J., Mazón, G., Dubbels, B.L., Bazylinski, D.A. & Barbé, J. (2003). Characterization of a new LexA binding motif in the marine magnetotactic bacterium strain MC-1. *J Bacteriol* **185**, 4471-4482.

Galhardo, R.S., Rocha, R.P., Marques, M.V. & Menck, C.F.M. (2005). An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*. *Nucleic Acids Res* **33**, 2603-2614.

Green, M.R. & Sambrook, J. (2012). *Molecular Cloning: a Laboratory Manual*, 4th edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Hare, J.M., Perkins, S.N. & Gregg-Jolly, L.A. (2006). A constitutively expressed, truncated *umuDC* operon regulates the *recA*-dependent DNA damage induction of a gene in *Acinetobacter baylyi* strain ADP1. *Appl Environ Microbiol* **72**, 4036-4043.

Hare, J.M., Adhikari, S., Lambert, K.V., Hare, A.E. & Grice, A. (2012). The *Acinetobacter* regulatory UmuDAb protein cleaves in response to DNA damage with chimeric LexA/UmuD characteristics. *FEMS Microbiol Lett* **334**, 57-65.

Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K., Choi, S., Ohtsubo, E., Baba, T., Wanner, B.L. & other authors (2006). Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol Syst Biol* **2**, 2006 0007.

Ippoliti, P.J., DeLateur, N.A., Jones, K.M. & Beuning, P.J. (2012). Multiple strategies for translesion synthesis in bacteria. *Cells* **1**, 799-831.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100**, 14339-14344.

Kitagawa, Y., Akaboshi, E., Shinagawa, H., Horii, T., Ogawa, H. & Kato, T. (1985). Structural analysis of the *umu* operon required for inducible mutagenesis in *Escherichia coli*. *Proc Natl Acad Sci USA* **82**, 4336-4340.

Kivisaar, M. (2010). Mechanisms of stationary-phase mutagenesis in bacteria: mutational processes in pseudomonads. *FEMS Microbiol Lett* **312**, 1-14.

Li, L.L., Malone, J.E. & Iglewski, B.H. (2007). Regulation of the *Pseudomonas aeruginosa* quorum-sensing regulator VqsR. *J Bacteriol* **189**, 4367-4374.

Luo, Y., Pfuetzner, R.A., Mosimann, S., Paetzel, M., Frey, E.A., Cherney, M., Kim, B., Little, J.W. & Strynadka, N.C. (2001). Crystal structure of LexA: a conformational switch for regulation of self-cleavage. *Cell* **106**, 585-594.

Nohmi, T. (2006). Environmental stress and lesion-bypass DNA polymerases. *Annu Rev Microbiol* **60**, 231-253.

Patel, M., Jiang, Q., Woodgate, R., Cox, M.M. & Goodman, M.F. (2010). A new model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. *Crit Rev Biochem Mol Biol* **45**, 171-184.

Petrova, V., Chitteni-Pattu, S., Drees, J.C., Inman, R.B. & Cox, M.M. (2009). An SOS inhibitor that binds to free RecA protein: the PsiB protein. *Mol Cell* **36**, 121-130.

Preston, M.J., Seed, P.C., Toder, D.S., Iglewski, B.H., Ohman, D.E., Gustin, J.K., Goldberg, J.B. & Pier, G.B. (1997). Contribution of proteases and LasR to

497 the virulence of *Pseudomonas aeruginosa* during corneal infections. *Infect Immun*
498 **65**, 3086-3090.
499
500 **Qi, Y., Patra, G., Liang, X., Williams, L.E., Rose, S., Redkar, R.J. & DelVecchio,**
501 **V.G. (2001).** Utilization of *rpoB* gene as a specific chromosomal marker for real-
502 time PCR detection of *Bacillus anthracis*. *Appl Environ Microbiol* **67**, 3720–3727.
503
504 **Ramírez-Díaz, M.I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-**
505 **García, J. & Cervantes, C. (2008).** Mechanisms of bacterial resistance to
506 chromium compounds. *Biometals* **21**, 321-332.
507
508 **Ramírez-Díaz, M.I., Díaz-Magaña, A., Meza-Carmen, V., Johnstone, L.,**
509 **Cervantes, C. & Rensing, C. (2011).** Nucleotide sequence of *Pseudomonas*
510 *aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal
511 resistance genes. *Plasmid* **66**, 7-18.
512
513 **Sutton, M.D., Kim, M. & Walker, G.C. (2001).** Genetic and biochemical
514 characterization of a novel *umuD* mutation: insights into a mechanism for UmuD
515 self-cleavage. *J Bacteriol* **183**, 347-357.
516
517 **Tark, M., Tover, A., Tarassova, K., Tegova, R., Kivi, G., Horak, R. & Kivisaar,**
518 **M. (2005).** A DNA polymerase V homologue encoded by TOL plasmid pWW0
519 confers evolutionary fitness on *Pseudomonas putida* under conditions of
520 environmental stress. *J Bacteriol* **187**, 5203-5213.

Walker, G.C. (1984). Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. *Microbiol Rev* **48**, 60-93.

Watanabe, S. & Takada, Y. (2004). Amino acid residues involved in cold adaptation of isocitrate lyase from a psychrophilic bacterium, *Colwellia maris*. *Microbiology* **150**, 3393-3403.

West, S.E., Schweizer, H.P., Dall, C., Sample, A.K. & Runyen-Janecky, L.J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**, 81-86.

Figure legends

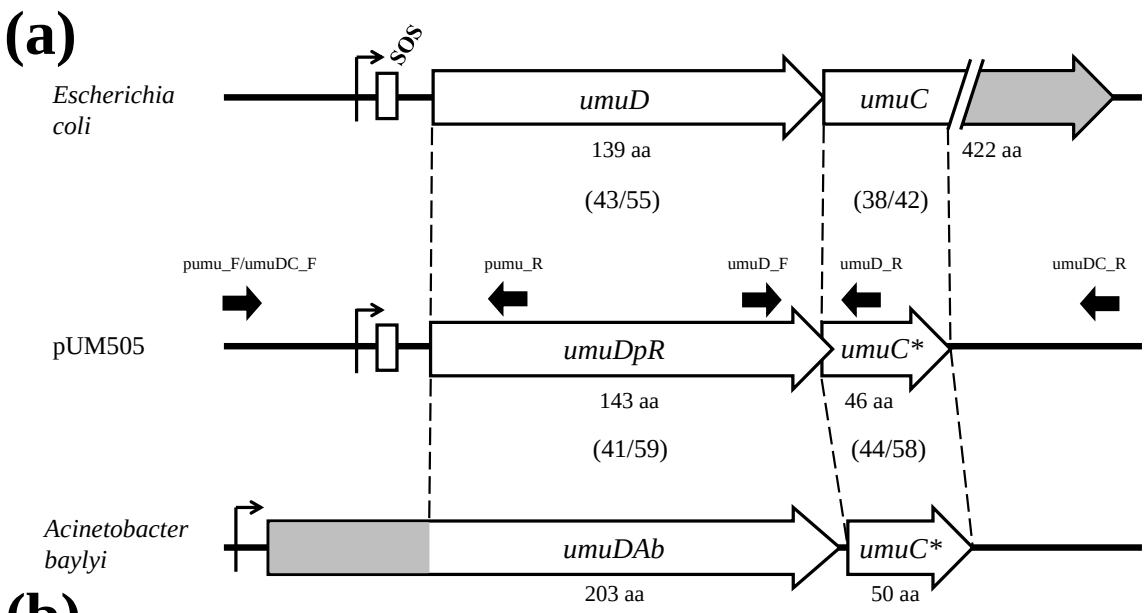
Figure 1. (a) Comparison of *umuDC* operons. Coding regions of *umuDC* genes from *Escherichia coli* K12, plasmid pUM505, and *Acinetobacter baylyi* ADP1 are indicated by large open arrows, with the size of predicted polypeptides (aa) indicated below; *umuC** corresponds to truncated *umuC* genes. The location of putative promoter sequences is marked by (↱) and SOS boxes are indicated by small rectangles. Percentages of amino acid sequence identity/similarity of the compared proteins are indicated in parentheses; shaded areas were not considered for the alignments. Black arrows signal the locations of primers, as described in the text. Information on the *umuDC* genes of *E. coli* and *A. baylyi* were drawn from Kitagawa *et al.* (1985) and Hare *et al.* (2006), respectively. **(b) Multiple sequence alignment of UmuD homologs**. Alignments include sequences of UmuDpR from pUM505 plasmid; LexA Paer, *Pseudomonas aeruginosa* PAO1; LexA Ecol, *Escherichia coli* K12; UmuDAb Abau, *Acinetobacter baumannii* ATCC 17978; UmuDAb Abay, *A. baylyi* ADP1; UmuD Ecol, *E. coli* K12; RumA Pret, *Providencia rettgeri* R391 plasmid; MucA Smar, *Serratia marcescens* R471a plasmid; and MucA Styp, *Salmonella typhimurium* R394 plasmid. Shaded black and grey areas correspond to identical and similar residues, respectively. The (Ala/Cys)-Gly cleavage site residues and Ser-Lys active site residues are indicated by black and white circles, respectively (Luo *et al.,* 2001). The Leu-Arg motif that is required for UmuD self-cleavage in *E. coli* is indicated by a rectangle (Sutton *et al.,* 2001).

Figure 2. Transcriptional analysis of *umuDpR-umuC genes. (a)** Reverse transcription (RT)-PCR products obtained using total RNA of *P. aeruginosa* PAO1 (pUM505) and primers that amplify a fragment containing the *umuDpR-umuC** overlap region (Fig. 1a) in the presence (lane 1) or absence (lane 2) of Mitomycin C (MMC) (1 µg ml^{-1}). Positive and negative controls (lanes 3 and 4) were obtained using plasmid DNA and total RNA as templates, respectively. Amplified fragments were separated in 1.5% agarose gel. M, Molecular size markers; sizes of bands (base pairs) are indicated to the left. **(b)** Alignment of the putative *umuDpR* promoter and the consensus Sigma factor-70 type promoter. Consensus sequences are indicated in bold and the nucleotides conserved are underlined. A putative SOS box sequence from *Escherichia coli* is also indicated by a rectangle and the consensus (Fernández de Henestrosa *et al.,* 2003) is shown above the *umuDpR* sequence; the nucleotides conserved are indicated in bold. **(c)** β-galactosidase activities of *P. aeruginosa* PAO1 (pLP_umuDC). Cultures in the exponential growth phase with no additions (white bars) or with MMC (0.2 µg ml^{-1}; black bars); incubation proceeded for the indicated times and β-galactosidase activities (expressed as Miller units) were measured as described in Methods. Data represent values from three independent assays, with Standard error bars shown.

Figure 3. Susceptibility to Mitomycin C (MMC) by bacterial strains. Cultures were grown in Nutrient broth for 18 h at 37°C with shaking with the indicated concentrations of MMC and OD_{600} was measured. **a)** (○), *Pseudomonas aeruginosa* PAO1 (pUCP20); (■), PAO1 (pUC_umuDC); (□), PAO1 (pUC_umuD); **b)** (○), *P. aeruginosa* PAO1; (●) PW6037 *lexA*$^{-}$ mutant; (□) PW6037

(pUC_umuD). Data shown are means of three assays in duplicate with Standard error bars shown.

Figure 4. Effect of UmuDpR on the expression of *P. aeruginosa* PAO1 SOS genes. Cultures were grown in Nutrient broth to the mid-exponential growth phase and total RNA was isolated and processed as described under Methods. Relative expression of each gene corresponds to wild-type PAO1 (WT), PW6037 *lexA*$^{-}$ mutant, or PW6037 transformed with the pUC_umuD plasmid (*lexA*$^{-}$*/umuDpR*). Values represent the means of three independent determinations in duplicate with Standard error bars shown, normalized with respect to transcription of the *rpoB* gene, calculated as described in the Methods section.



(b)

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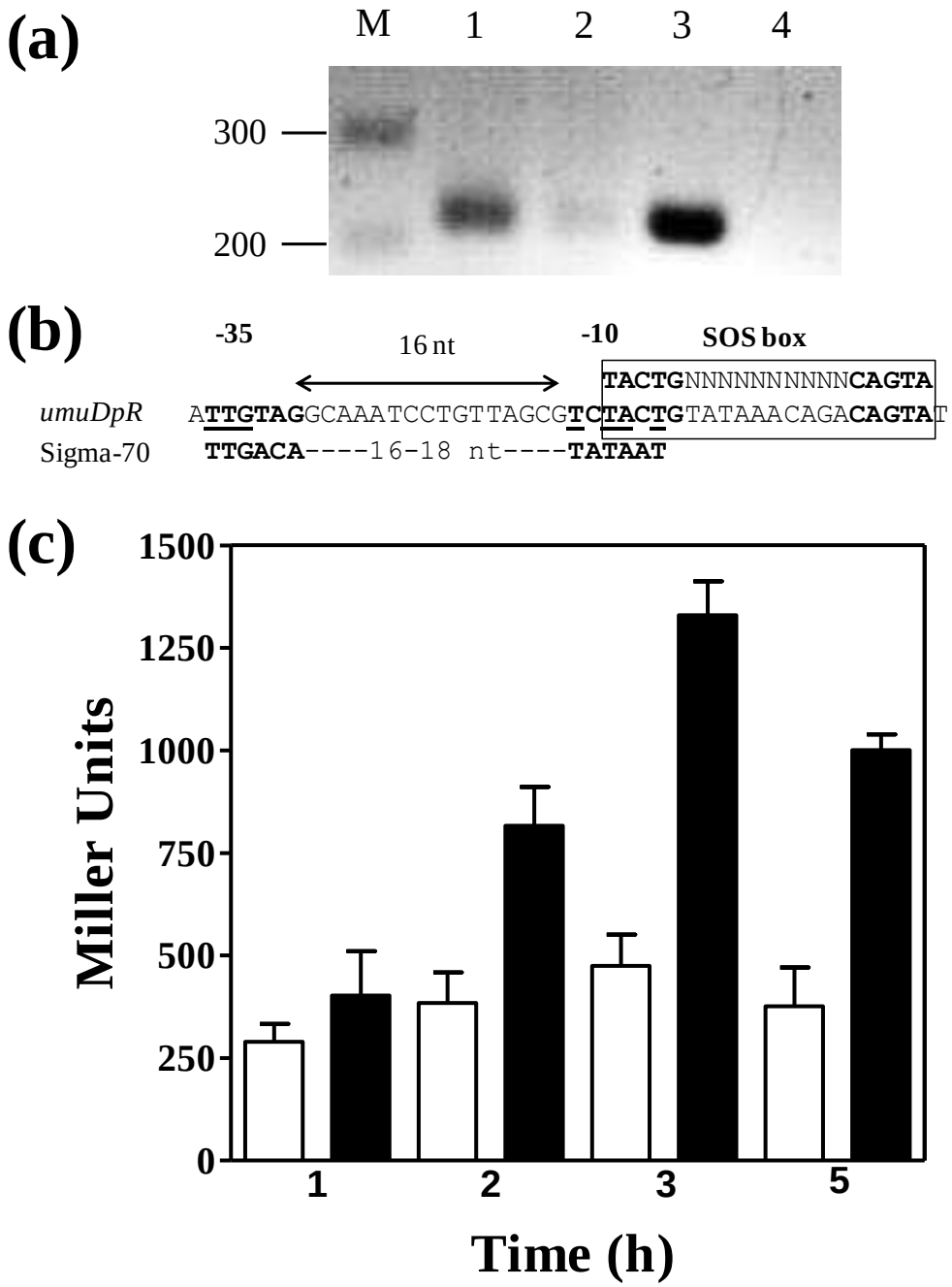
UmuDpR       ----------------------------------------MP---VTILGP----------------VAGSGASLRLYS---------
LexA Paer    MQKLTPRQAEILSFIKRCLEDHGFPPTRAEIAQELGFKSPNAAEEHLKALARKG---------AIEMTPGASRGIRIPGFEPHAANDDEG
LexA Ecol    MKALTARQQEVFDLIRDHISQTGMPPTRAEIAQRLGFRSPNAAEEHLKALARKG---------VIEIVSGASRGIRLLQEE------EEG
UmuDAb Abau  ---MPKKKEFEHGGARENAGRKAQFNEPTKVIRVPESQVNFIKNWLLNNVKTNNQTDFTSKLKIQQVHPNNDKIYHIPLAT---------
UmuDAb Abay  ---MSQQNKNEHGGARTNAGRKAKYQEPTKVIRVPESQVAFIKRWLLDNVKTDNLIDFNSALKVQAIQPNPTKIYQIPLAT---------
UmuD Ecol    ----------------------------------------MLFIK---PADLRE----------------IVT-----FPLFS---------
RumA Pret    ----------------------------------------MSVSLIG---RSGVLA----------------FIKAKRLRIPLFM---------
MucA Smar    ----------------------------------------MA---LDLVYP----------------RPSVEGDALPFFA---------
MucA Styp    ----------------------------------------MS---LKPMKS----------------APDTK-ETIPFFG---------

UmuDpR       ----SKVSAGFPSPAQDHLEREISLDEILQIRAPHTYLVRAGGDSMIRAGIHDGDLMVVDRSREAEFGDIVIAAVNGEFTVKRLAKDG--
LexA Paer    LPVIGRVAAGAPILAEQNIEESCRINPAFFN-PRADYLLRVRGMSMKDIGILDGDLLAVHVTREARNGQVVVARIGEEVTVKRFKREG--
LexA Ecol    LPLVGRVAAGFPLLAQQHIEGHYQVDPSLFK-PNADFLLRVSGMSMKDIGIMDGDLLAVHKTQDVRNGQVVVARIDDEVTVKRLKKQG--
UmuDAb Abau  ----ERVAAGFPSPAQDDIEQALDLNEYLIRNENATFIVKANSLSMLDAGIDIDDPLIVDRSIPAKSGDIVIALIDNDFTVKRLIIDTQF
UmuDAb Abay  ----ERVAAGLPSPAQEHVEQSLDLNEYLVRNENATFIVKANSLSMLDAGIDIDDPLIVDRSITAKAGDIVIAMVDNEFTVKRLMIDHHF
UmuD Ecol    ----DLVQCGFPSPAADYVEQRIDLNQLLIQHPSATYFVKASGDSMIDGGISDGDLLIVDSAITASHGDIVIAAVDGEFTVKKLQLR---
RumA Pret    ----ERVSAGFPSPAQDYVEQTLDLNELCIKRPAATFFVRVEGDSMIDAGIHPDDILVVDRSVQAEHGDIVIAGIHGELTVKELQLR---
MucA Smar    ----EPVAAGFPSPAAGYEANELNLHDYCVRHPAATYFLRVSGDSMRDARIHDGDVLVVDRAEEPRHGSIVIASIDNEFTVKQLQLR---
MucA Styp    ----ELVPAGFPSPAAGWEEAELNLHTLVVTHPASTYFLRVTGDSMQDARIHSGDVLVVDRSETPEQGSIVVASIDNEFTVKKLILR---

UmuDpR       --SQVVLRAENPRYAPRYILEGD-ELLVWGVVRYSVRCHEHG-- 143
LexA Paer    --SKVWLLAENPEFAPIEVDLKEQELIIEGLSVGVIRR------ 204
LexA Ecol    --NKVELLPENSEFKPIVVDLRQQSFTIEGLAVGVIRNGDWL-- 202
UmuDAb Abau  QPPKVWLKAENPDYQNIYIEEGQ-ELVIWGVVTYNLKRMR---- 203
UmuDAb Abay  HPPKVWLKAENPDFQNIYIDEGQ-ELVIWGVVTYNLKPMR---- 203
UmuD Ecol    --PTVQLIPMNSAYSPITISSED-TLDVFGVVIHVVKAMR---- 139
RumA Pret    --PCVKLIPRNQAYEPIHIPEGT-ELEIFGVVTNVVRNMRRKS- 149
MucA Smar    --PYPCLMPLNPAFPPIRFDPEA--LQIWGVVTFTVMRHRPCTP 144
MucA Styp    --PRPCLMPMNPAYPPIYFDPESNDVEIWGVVTYSLMKHKKCMA 145
```

Figure 1.



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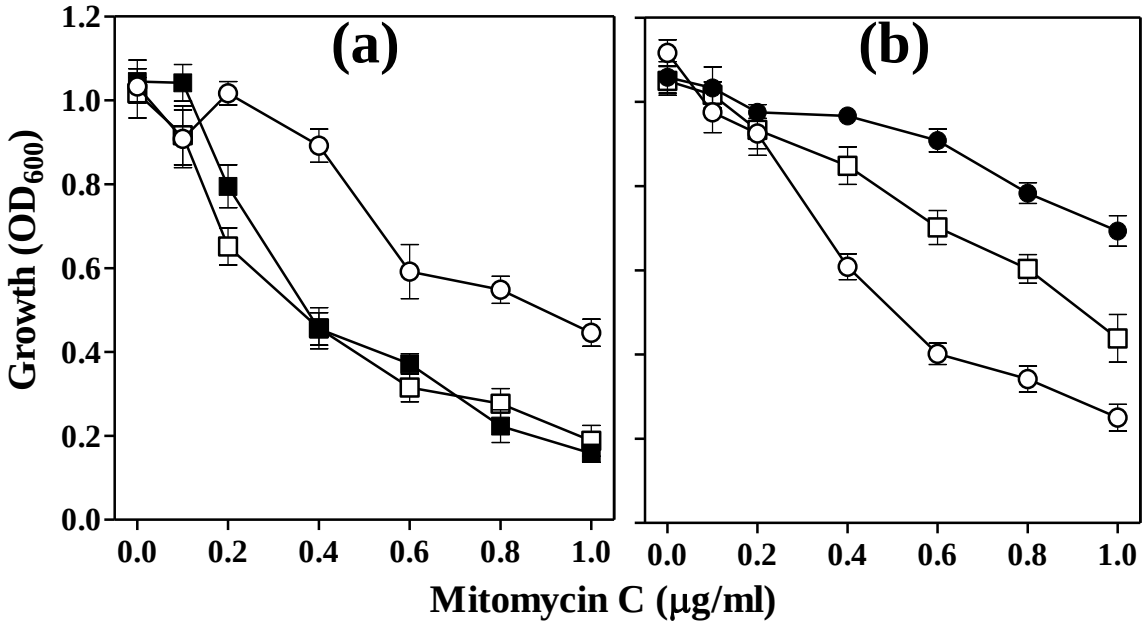
594 Figure 2.



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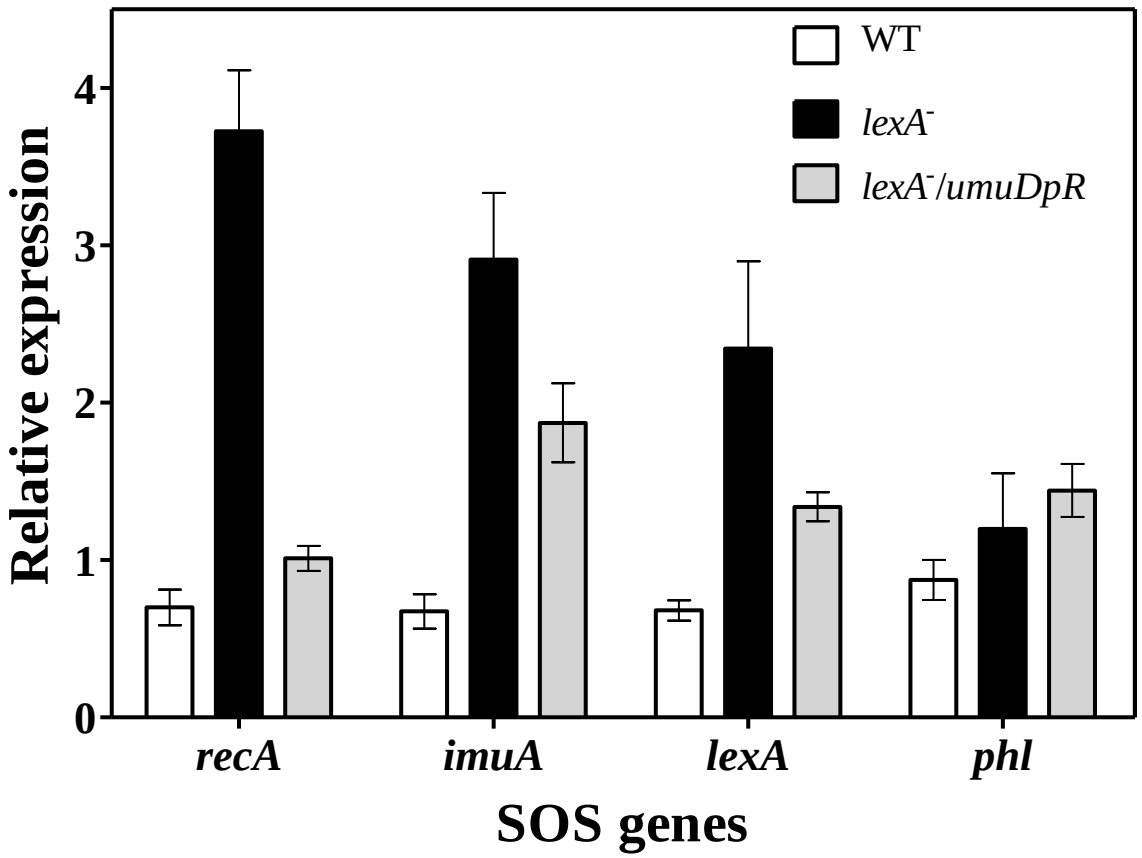
596 Figure 3.



597

598 Figure 4.

599 **Supplementary material**

600 Table S1. Primers used in this study.

Gene/ORF	Primer name	Sequence (5´ → 3´)	Tm (°C)	Amplicon size (bp)
RT-PCR				
*umuDpR-umuC**	umuD_F	GCTGAGCCAGGAGACATCGTAATTG	60	222
	umuD_R	CTGTTGAAGCTTATCAGGGCGATGG †	60	
LacZ fusions				
umuDpR	pumu_F	GAAGAATTCCGACCTGTGTTGAAG ‡	56	378
	pumu_R	CGGATCCTCATCAAGCGAGATCTC §	59	
Gene Cloning				
*umuDpR-umuC**	umuDC_F	GAAGGATCCCGACCTGTGTTGAAG §	59	1147
	umuDC_R	CCATCTGCCTGACAAGCTTCATTCC †	60	
Overexpression				
umuDpR	UmuD_F	GTTTGGATCCATGCCCGTTAC §	55	459
	UmuD_R	GATGGGAATTCCAGCCATGCTC ‡	55	
***lexA*⁻ mutant** &				
lexA	32620F.f	CCTTCTTCGAACCGAAGTGA	60	1157
	32620F.r	CGAAAGTTCGCTAAAGGCAG	60	
qRT-PCR				
phl/PA0069	F_PA0069	AGTCGCCGGACCTCCCTTT	68	82
	R_PA0069	GGCCGCGCATAGCAGTAG	65	
imuA/PA0671	F_PA0671	GCATGCCCTGAGCGAAATC	64	70
	R_PA0671	GAGGGTCGGCATCAGTAGTTG	65	
lexA/PA3007	F_PA3007	GCCGAACAGAACATCGAGGAATC	65	79
	R_PA3007	GCACGCGCAACAGGTAGT	66	
recA/PA3617	F_PA3617	AGGCGCTGCGCAAGATCAC	68	74
	R_PA3617	CATGCGGATCTGGTTGATGAA	63	
rpoB	rpoB_F	GCCGCGATCAAGGAGTTC	63	70
	rpoB_R	CGGAAAGCGGGTTGTTCTG	64	
EMSA				
phl/PA0069	P0069_F	CGAATGCGTGGCCTG	54	386
	P0069_R	CTCGGTGAGCTGGATCG	55	

imuA/PA0671	P0671_F	GACATAGCGTGCCCAG	53	408
	P0671_R	GTCGGCATCAGTAGTTG	50	
lexA/PA3007	P3007_F	GTTTCCGACTGGGCCATG	56	395
	P3007_R	CGCCTTGAGGTGCTCC	56	
recA/PA3617	P3617_F	CTGGTCAGTGAGCGCTG	56	392
	P3617_R	CGATGTCCAGACCCAGG	55	

†*Hin*dIII, ‡ *Eco*RI and § *Bam*HI endonuclease restriction sites are underlined in the indicated primers.

& Primers reported in *P. aeruginosa* Mutant Library (http://www.gs.washington.edu/labs/manoil/libraryindex.htm).

606 Table S2. *Pseudomonas aeruginosa* PAO1 SOS genes †

607

Gene/ORF	Predicted function	SOS box ‡	Position §
phl/PA0069	DNA repair photolyase	CTGTATCCATATACAG	20
imuA/PA0671	Recombinase	CTGTATTTACATACAG	153
lexA/PA3007	Repressor	CTGGATAAAAACACAG	9
recA/PA3617	Recombinase	CTGTCTACTTATACAG	43

608

609 † Information of SOS boxes sequences were drawn from Cirz *et al.* (2006).

610 ‡ Conserved nucleotides with respect to the SOS box consensus sequence from *E. coli* (Fernández

611 de Henestrosa *et al.*, 2003) are underlined.

612 § Number of nucleotides between the predicted regulatory sequence and the start codon of the gene.

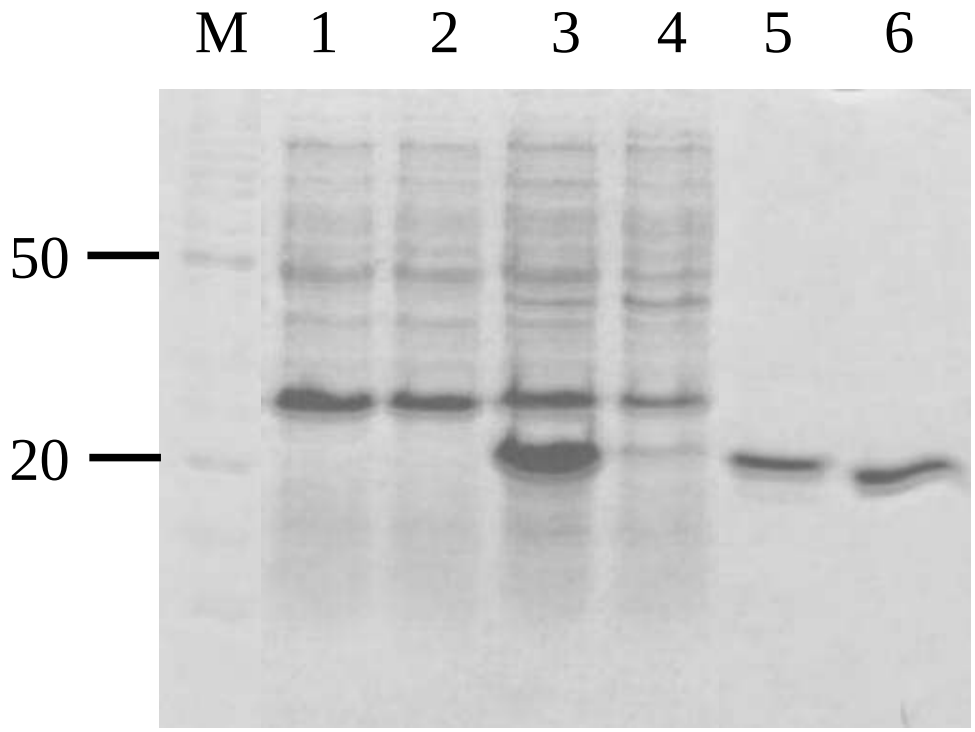
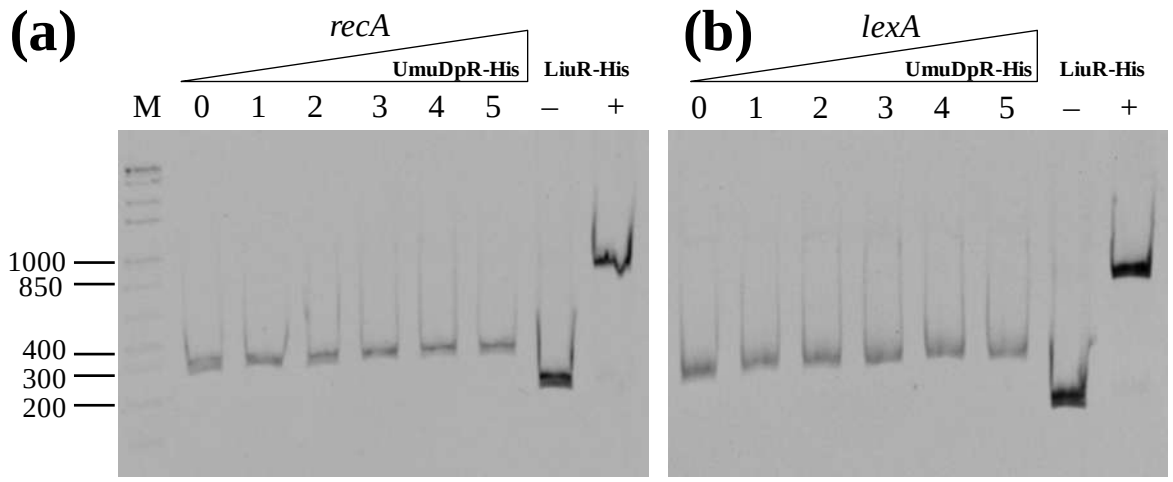


Figure S1. Overexpression and purification of recombinant UmuDpR-His protein. SDS-PAGE analysis of lysates from *E. coli* BL21-Codon-Plus(DE3)-RP cells carrying empty pTrcHis2A vector or pTrcUmuD-His untreated (lanes 1 and 4, respectively) or induced with 0.1 mM IPTG (lanes 2 and 3, respectively). Lanes 5 and 6 show proteins UmuDpR-His and LiuR-His (positive control for EMSA), respectively, after purification by Ni-NTA affinity chromatography. M, molecular mass markers shown to the left in kilodaltons.



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623

624 **Figure S2. Binding of UmuDpR to the 5´ regions of SOS genes**. Purified DNA
625 fragments (100 ng) containing the 5´ regions of the SOS genes *recA* **(a)** and *lexA*
626 **(b)** were each mixed and incubated with the indicated amounts of purified
627 UmuDpR-His protein (µg) as described in the Methods section. For EMSA, free
628 DNA and protein-DNA complexes were resolved by 6 % native PAGE and stained
629 with ethidium bromide. Gel retardation by control LiuR-His protein (0.5 µg) is
630 shown in the lanes marked as (+). M, molecular size markers shown to the left in
631 base pairs.

CAPÍTULO III.

Análisis estructural y funcional del gen *pdi* del plásmido pUM505 de *Pseudomonas aeruginosa*

Análisis estructural y funcional del gen *pdi* del plásmido pUM505 de *Pseudomonas aeruginosa*

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Resumen

El plásmido pUM505 de *Pseudomonas aeruginosa* posee el gen *pdi,* que se predice que codifica a una probable disulfuro isomerasa de proteínas, enzimas que cataliza la formación y ruptura de enlaces disulfuro entre residuos de cisteína en proteínas. El objetivo de este trabajo es analizar la estructura y función del gen *pdi* del plásmido pUM505. El gen *pdi* de pUM505 posee en su región reguladora un posible promotor reconocido por el factor sigma S. Mediante fusiones transcripcionales de esta región se determinó que el promotor del gen *pdi* es funcional, con una mayor expresión en la fase estacionaria, y ésta es regulada de manera dependiente del factor sigma S. Por lo tanto, la proteína Pdi de pUM505 podría requerirse para cambios adaptativos que ocurren en esta etapa del crecimiento bacteriano. Un análisis *in silico* de la proteína predicha Pdi mostró que ésta tiene un dominio estructural "plegamiento tiorredoxina" con dos motivos conservados: el sitio activo $C_{66}XXC_{69}$ y el motivo "asa cis-prolina", típicos de las proteínas DsbAs de las γ-proteobacterias. También posee un par de cisteínas conservadas (C_{111} y C_{157}), propias de la subclase α-DsbA presente en α-proteobacterias. Además, Pdi posee un residuo conservado treonina (T_{183}) característico de las disulfuro isomerasas (DsbC/DsbG) de *Escherichia coli*. Por lo tanto, Pdi podría realizar la función de oxidación/reducción e isomerización de enlaces disulfuro en proteínas de *P. aruginosa*.

Abstract

The *Pseudomonas aeruginosa* plasmid pUM505 contains the *pdi* gene that encodes a protein similar to putative protein disulfide isomerases (Pdi), enzymes that catalyze formation of protein disulfide bonds. The objective of this work was to analyze the

structure and function of pUM505 *pdi* gene. The putative regulatory region of the *pdi* gene possesses a potential promoter related with S type sigma factor. Transcriptional fusions of this region revealed that the *pdi* gene promoter is functional, with maximal expression in the stationary growth phase, and this is dependent on the S-type sigma factor. Therefore, pUM505 Pdi protein may be required for adaptive changes that occur during this stage of bacterial growth. An *in silico* analysis of predicted protein Pdi showed that this has a structural “thioredoxin fold” domain with two conserved motifs: $C_{66}XXC_{69}$, a catalytic site, and the “cis-proline loop” motif, present in oxidoreductases DsbAs from γ-proteobacteria. Also, Pdi has two conserved cysteines (C_{111} and C_{157}), typical of the subclase α-DsbA present in α-proteobacteria. Furthermore, Pdi possesses a conserved threonine residue (T_{183}) characteristic of disulfide isomerases such as (DsbC/DsbG) from *Escherichia coli*. Therefore, Pdi could have the function of oxidation/reduction and isomerization of disulfide bonds of proteins from *P. aeruginosa*.

Introducción

El plásmido pUM505 posee el gen *pdi*, que se predice que codifica a una disulfuro isomerasa de proteínas. En la mayoría de las bacterias Gram-negativas la formación de enlaces disulfuro en las proteínas es realizada por miembros de la familia de enzimas óxido-reductasa tiol/disulfuro Dsb (por sus siglas en inglés *di*sulfide *b*ond), que catalizan la oxidación del grupo tiol de las proteínas y la reducción e isomerización de enlaces disulfuro (Chivers *et al.* 1997). Estas reacciones no sólo son importantes para la integridad de las proteínas sino también para la función de algunas enzimas (Chivers *et al.* 1997); entre éstas se encuentran factores de

virulencia, los cuales requieren la formación de enlaces disulfuro para su estabilidad y función (Halili *et al.* 2015).

Las proteínas Dsb pertenecen a la superfamilia de las tiorredoxinas, las cuales poseen un dominio estructural conocido como plegamiento tiorredoxina, que contiene dos motivos conservados: el sitio activo CXXC y el motivo denominado “asa cis-prolina” (Martin 1995). Estas proteínas son importantes para el estilo de vida de la bacteria y juegan un papel principal en la virulencia de diversas especies bacterianas. La deleción del gen *pdi* de la isla de patogenicidad PAPI-1 de *P. aeruginosa* PA14, cepa significativamente virulenta causó un fenotipo de virulencia atenuada en *Arabidopsis* y en ratón (He *et al.* 2004).

Las proteínas parecidas a las tiorredoxinas presentes en los plásmidos conjugativos se relacionan con el hecho que estas proteínas podrían desempeñan un papel esencial en la conjugación, sirviendo como mediadores en el plegamiento y/o el ensamblaje de las proteínas que forman el poro de conjugación (Hemmis y Schildbach 2013). El objetivo de este trabajo fue establecer la función del gen *pdi* del plásmido pUM505.

Materiales y métodos

Cepas bacterianas y condiciones de cultivo

P. aeruginosa PAO1 (Li *et al.* 2007) y *Escherichia coli* W3110 (Hayashi *et al.* 2006) son cepas estándar protótrofas, empleadas como hospedero de los plásmidos recombinantes. La cepa PW7152 (*rpoS*::ISlacZ/bp) mutante derivada de PAO1 (obtenida de *P. aeruginosa* Mutant Library; Jacobs *et al*. 2003; Donada por el Dr. Campos-García) se utilizó para los análisis de la expresión transcripcional del gen

pdi. Se emplearon los medios de cultivo caldo nutritivo (CN) o caldo Luria-Bertani (LB; para el medio sólido se adicionó 1.5% de agar bacteriológico) (Green y Sambrook 2012). Se adicionó ampicilina (100 µg ml^{-1}) o carbenicilina (400 µg ml^{-1}) cuando fue necesario. Las células fueron crecidas rutinariamente a 37°C con agitación durante toda la noche y el crecimiento fue monitoreado como densidad óptica (DO) a 600 nm (DO_{600}) con un espectrofotómetro.

Técnicas genéticas y análisis de secuencias

Los procedimientos moleculares se realizaron de acuerdo a métodos estándar (Green y Sambrook 2012). Los procesos de clonación de las construcciones se verificaron por secuenciación del DNA realizado en el departamento de Genética, Cinvestav, Irapuato, México. Los alineamientos de la secuencia de proteínas se calcularon con el algoritmo CLUSTALW y el software BioEdit Sequence Alignment Editor. Las secuencias similares se buscaron en la base de datos de proteínas empleando el programa blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). La secuencia de probables promotores se buscó empleando el programa Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). La predicción de la estructura terciaria de la proteína Pdi se construyó empleando el programa Swiss Model (http://swissmodel.expasy.org/).

Clonación del gen *pdi*

El gen *pdi* se obtuvo por PCR utilizando como molde el DNA del plásmido pUM505, empleando los oligonucleótidos directo pdi_F (5´-GAATGAGAGCTCTGAATTCGCTGGAC-3´) y reverso pdi_R (5´-

CACCTACGAGTTGAAGCTTTCCTGC-3´) (Fig. 1a), con los sitios de restricción *Eco*RI y *Hin*dIII (subrayados), respectivamente. El fragmento amplificado se purificó y se clonó en el vector pJET1.2/blunt (Fermentas). El plásmido recombinante pJET_pdi se transfirió por electroporación a *E. coli* W3110 y las transformantes se seleccionaron en placas de agar LB con ampicilina. El fragmento de DNA digerido con las enzimas *Eco*RI y *Hin*dIII se subclonó en el vector binario de expresión *Escherichia/Pseudomonas* pUCP20 (West *et al*. 1994). El plásmido recombinante pUC_pdi se transfirió por electroporación a *P. aeruginosa* PAO1 y las transformantes se seleccionaron en placas de agar LB con carbenicilina.

Fusión transcripcional

Se obtuvo un fragmento de DNA de 621 pb que contiene la probable región promotora del gen *pdi* del plásmido pJET_pdi, empleando las endonucleasas *Eco*RI y *Bam*HI. El fragmento de DNA se purificó y se clonó en los sitios correspondientes del vector pLP170, que posee el gen reportero *lacZ* sin promotor (Preston *et al.* 1997). El plásmido pLP_pdi se transfirió por electroporación a *P. aeruginosa* PAO1 y las transformantes se seleccionaron en placas de agar LB con carbenicilina. La actividad de β-galactosidasa se determinó en células permeabilizadas empleando el sustrato cromogénico ONPG (Sigma), como se describe en Acosta-Navarrete *et al.* (2014). La actividad enzimática se corrigió restando los valores obtenidos de las células que contienen el vector pLP170 sin inserto.

Pruebas de susceptibilidad

Las bacterias crecidas fueron diluidas 1:100 en tubos con 4 ml de CN con cantidades crecientes de Paraquat (Sigma), Menadiona (Sigma) o K_2CrO_4 (J.T. Baker). Los cultivos fueron incubados por 18 h a 37°C con agitación y el crecimiento se midió como DO_{600}.

Resultados y discusión

La expresión del gen *pdi* de pUM505 es regulada en la fase estacionaria

Mediante el análisis de la secuencia de nucleótidos de pUM505 se identificó el gen *pdi*, que codifica una probable disulfuro isomerasa de proteínas (Pdi) de 219 aa (Ramírez-Díaz *et al*. 2011) (Fig. 1a). El análisis de la posible región reguladora del gen *pdi* mostró que presenta un promotor potencial con cajas consenso -35, -13 y -10, relacionadas con secuencias reconocidas por el factor sigma S (Fig. 1b). Esto sugiere que la expresión de *pdi* podría ocurrir en la fase estacionaria, etapa en la que actúa dicho factor transcripcional (Hengge-Aronis, 2002). Para confirmar esta hipótesis, la posible región reguladora de *pdi* de pUM505 se clonó río arriba del gen reportero *lacZ*, carente de promotor, del vector pLP170 y se evaluó la actividad de β-galactosidasa. Los cultivos de la cepa silvestre *P. aeruginosa* PAO1 (pLP_pdi) mostraron una actividad significativa de β-galactosidasa (~ 125 Unidades Miller) a las 2 h del ensayo (Fig. 1c, barras blancas), indicando que el promotor del gen *pdi* es funcional. Cuando el cultivo alcanzó la fase exponencial tardía (4 h), se observó un incremento de la actividad de β-galactosidasa de 3.6 veces con respecto a la actividad presente a las 2 h (Fig. 1c), presentando el nivel máximo de actividad (8.4 veces) en la fase estacionaria tardía (8 h) (Fig. 1c). Estos resultados sugieren que la región promotora de *pdi* es regulada por algún factor de la fase de crecimiento. Este

resultado concuerda con lo reportado para el promotor del gen *dsbA*, homólogo de *pdi*, de *Salmonella typhimurium* que es regulado por factores relacionados con la fase de crecimiento, tales como cambios del pH, niveles de oxígeno y limitación de nutrientes (Goecke *et al.* 2002).

Para confirmar que la expresión de *pdi* de pUM505 es regulada por algún componente de la fase de crecimiento, el plásmido pLP_pdi se transfirió a la cepa PW7152, una mutante derivada de PAO1 afectada en el gen *rpoS* el cual codifica el factor transcripcional sigma RpoS que actúa en la fase estacionaria. Cuando se comparó la actividad de β-galactosidasa de la región promotora de *pdi* de la cepa silvestre con la actividad de la mutante afectada en *rpoS*, el patrón de expresión fue similar en ambas cepas durante la fase exponencial (Fig. 1c). Sin embargo, durante la fase estacionaria hubo un decremento de la actividad de β-galactosidasa de alrededor de dos veces en la mutante afectada en *rpoS* con respecto a la cepa tipo silvestre (Fig. 1c). Esto indica que, al menos parcialmente, la regulación del gen *pdi* en la fase estacionaria es realizada por RpoS.

En resumen, el análisis de la expresión del gen *pdi* del plásmido pUM505 indica que éste posee un promotor funcional con una mayor expresión en la fase estacionaria de crecimiento el cual es regulado de una manera dependiente del factor sigma S, sugiriendo que Pdi de pUM505 podría requerirse para algunos cambios adaptativos que ocurren en esta etapa del crecimiento bacteriano.

Proteína Pdi del plásmido pUM505

La búsqueda blast permitió determinar que la proteína Pdi predicha presenta un rango de identidad del 93-95% con proteínas Pdi codificadas en el cromosoma de

diversos aislados clínicos de *P. aeruginosa*. En la Fig. 2 se muestra un alineamiento representativo con algunas de esas proteínas Pdi, entre ellas una proteína recientemente caracterizada como una oxidorreductasa y renombrada como DsbA2 de *P. aeruginosa* PA14 (Arts *et al.* 2013), la cual mostró un 95% de identidad. Mediante un alineamiento múltiple de la secuencia de aminoácidos de Pdi de pUM505 y sus homólogos de *P. aeruginosa* se identificó el probable sitio catalítico redox $C_{66}XXC_{69}$ y un par de cisteínas conservadas (C_{111} y C_{157}) (Fig. 2), características de las proteínas de la subclase α-DsbA (Kurz *et al*. 2009); así mismo se identificó el motivo “asa cis-prolina” (P_{184}) y el residuo conservado treonina (T_{183}) (Fig. 2). Éste último contribuye en la actividad redox y en la interacción con las proteínas sustrato además de participar en la actividad de isomerización (Kurz *et al*. 2009). Una treonina en la misma posición es característica de las disulfuro isomerasas tales como DsbC y DsbG de *E. coli* (Kurz *et al*. 2009).

Por otra parte, se realizó una predicción de la estructura terciaria de la proteína Pdi de pUM505, para lo cual se usó como molde la estructura de la proteína cristalizada DsbA2 de *P. aeruginosa* (4n30.1.A). En el modelo predicho de Pdi se localizó el dominio tiorredoxina (Trx) altamente conservado, que consiste en cuatro láminas beta rodeadas por tres hélices alfa (Fig. 3). Este dominio es típico en las proteínas de la superfamilia de las tiorredoxinas (Messens y Collet 2009), que incluye a las glutarredoxinas, tiorredoxinas, enzimas relacionadas con las glutatión peroxidasas y las proteínas DsbA y DsbC (Wouters *et al.* 2010). Además, en el modelo de Pdi de pUM505 se encontróxc el dominio hélice alfa (Fig. 3) que está presente en las proteínas DsbA (Martin 1995); en este dominio se localizan un par de residuos

adicionales de cisteína (C_{111} y C_{157}) (Fig. 3), con una función reguladora, que están conservadas en las proteínas de la subclase α-DsbA (Kurz *et al*. 2009).

El análisis estructural *in silico* de la proteína Pdi de pUM505 sugiere que esta proteína podría tener la capacidad de catalizar tanto la oxidación, reducción e isomerización de enlaces disulfuro en proteínas.

Análisis funcional del gen *pdi*

Miembros de la familia Dsb se inducen por la presencia de peróxido, agente generador de estrés oxidativo, en la cianobacteria *Synechocystis* (Li *et al.* 2004), posiblemente la proteína Pdi de pUM505 combate el estrés oxidativo. Para determinar si la proteína Pdi del plásmido pUM505 es capaz de contrarrestar el estrés oxidativo, el gen *pdi* se amplificó por PCR y se clonó en el vector pUCP20, obteniendo el plásmido recombinante pUC_pdi, el cual se transfirió a *P. aeruginosa* PAO1. Se realizaron ensayos de susceptibilidad de las transformantes a los agentes que causan estrés oxidativo paraquat, menadiona y cromato. Sin embargo, las transformantes expresando al gen *pdi* mostraron un crecimiento similar comparado con la cepa control *P. aeruginosa* PAO1 (pUCP20) (datos no mostrados); esto sugiere que *pdi* de pUM505 en las condiciones probadas no contrarresta el estrés oxidativo causado por estos compuestos, por lo que en este trabajo no se logró identificar la función de la proteína Pdi del plásmido pUM505.

Pdi podría tener una participación en la formación de enlaces disulfuro contribuyendo en el plegamiento de proteínas; algunas de éstas podrían ser factores de virulencia como se ha reportado para otras proteínas DsbAs (Miki *et al*. 2004).

Referencias

1. Acosta-Navarrete, Y. M., León-Márquez, Y. L., Salinas-Herrera, K., Jácome-Galarza, I. E., Meza-Carmen, V., Ramírez-Díaz, M. I. y Cervantes, C. (2014) Expression of the six chromate ion transporter homologues of *Burkholderia xenovorans* LB400. *Microbiology* **160**, 287-295.
2. Arts, I.S., Ball, G., Leverrier, P., Garvis, S., Nicolaes, V., Vertommen, D., Ize, B., Dufe, V.T., Messens, J., Voulhoux, R. y Collet, J.F. (2013) Dissecting the machinery that introduces disulfide bonds in *Pseudomonas aeruginosa*. *mBio*. **4**, e00912-13.
3. Chivers, P.T., Prehoda, K.E. y Raines, R.T. (1997) The CXXC motif: a rheostat in the active site. *Biochemistry* **36**, 4061-4066.
4. Goecke, M., Gallant, C., Suntharalingam, P., Martin, N.L. (2002) *Salmonella typhimurium* DsbA is growth-phase regulated. *FEMS Microbiol. Lett.* **206**, 229-234.
5. Green, M.R. y Sambrook, J. (2012) *Molecular Cloning: a Laboratory Manual*, 4th edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
6. Halili, M.A., Bachu, P., Lindahl, F., Bechara, C., Mohanty B., Reid, R.C., Scanlon, M.J., Robinson, C.V., Fairlie, D.P. y Martin J.L. (2015) Small molecule inhibitors of disulfide bond formation by the bacterial DsbA–DsbB dual enzyme system. *ACS Chem. Biol.* **10**, 957-964.
7. Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K., Choi, S., Ohtsubo, E., Baba, T., Wanner, B.L. y otros autores. (2006) Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol. Syst. Biol.* **2**, 2006 0007.

8. He, J., Baldini, R.L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N.T. *et al.* (2004) The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl. Acad. Sci. USA* **101**, 2530-2535.

9. Hemmis, C.W. y Schildbach, J.F. (2013) Thioredoxin-like proteins in F and other plasmid systems. *Plasmid* **70**, 168-189.

10. Hengge-Aronis, R. (2002) Stationary phase gene regulation: what makes an *Escherichia coli* promoter σS-selective? *Curr. Opin. Microbiol.* **5**, 591-595.

11. Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. y otros autores. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **100**, 14339-14344.

12. Kurz, M., Iturbe-Ormaetxe, I., Jarrott, R., Shouldice, S.R., Wouters, M.A., Frei, P., Glockshuber, R., O'Neill, S.L., Heras, B., Martin, J.L. (2009) Structural and functional characterization of the oxidoreductase alpha-DsbA1 from *Wolbachia pipientis*. *Antioxid. Redox Signal.* **11**, 1485-1500.

13. Li, H., Singh, A.K., McIntyre, L.M. y Sherman, L.A. (2004) Differential gene expression in response to hydrogen peroxide and the putative PerR regulon of *Synechocystis sp.* strain PCC 6803. *J. Bacteriol*. **186**, 3331-3345.

14. Li, L.L., Malone, J.E. y Iglewski, B.H. (2007) Regulation of the *Pseudomonas aeruginosa* quorum-sensing regulator VqsR. *J. Bacteriol.* **189**, 4367-4374.

15. Martin, J.L. (1995) Thioredoxin - a fold for all reasons. *Structure* **3**, 245-250.

16. Messens, J. y Collet, J.F. (2006) Pathways of disulfide bond formation in *Escherichia coli*. *Int. J. Biochem. Cell Biol*. **38**, 1050-1062.

17. Miki, T., Okada, N. y Danbara, H. (2004) Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the *Salmonell*a pathogenicity island 2 type III secretion system. *J. Biol. Chem.* **279**, 34631-34642.

18. Preston, M.J., Seed, P.C., Toder, D.S., Iglewski, B.H., Ohman, D.E., Gustin, J.K., Goldberg, J.B. y Pier, G.B. (1997) Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. *Infect. Immun.* **65**, 3086-3090.

19. Ramírez-Díaz, M.I., Díaz-Magaña, A., Meza-Carmen, V., Johnstone, L., Cervantes, C. y Rensing, C. (2011) Nucleotide sequence of *Pseudomonas aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal resistance genes. *Plasmid* **66**, 7-18.

20. West, S.E., Schweizer, H.P., Dall, C., Sample, A.K. y Runyen-Janecky, L.J. (1994) Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**, 81-86.

21. Wouters, M.A., Fan, S.W. y Haworth, N.L. (2010) Disulfides as redox switches: from molecular mechanisms to functional significance. *Antioxid. Redox Signal.* **12**, 53-91.

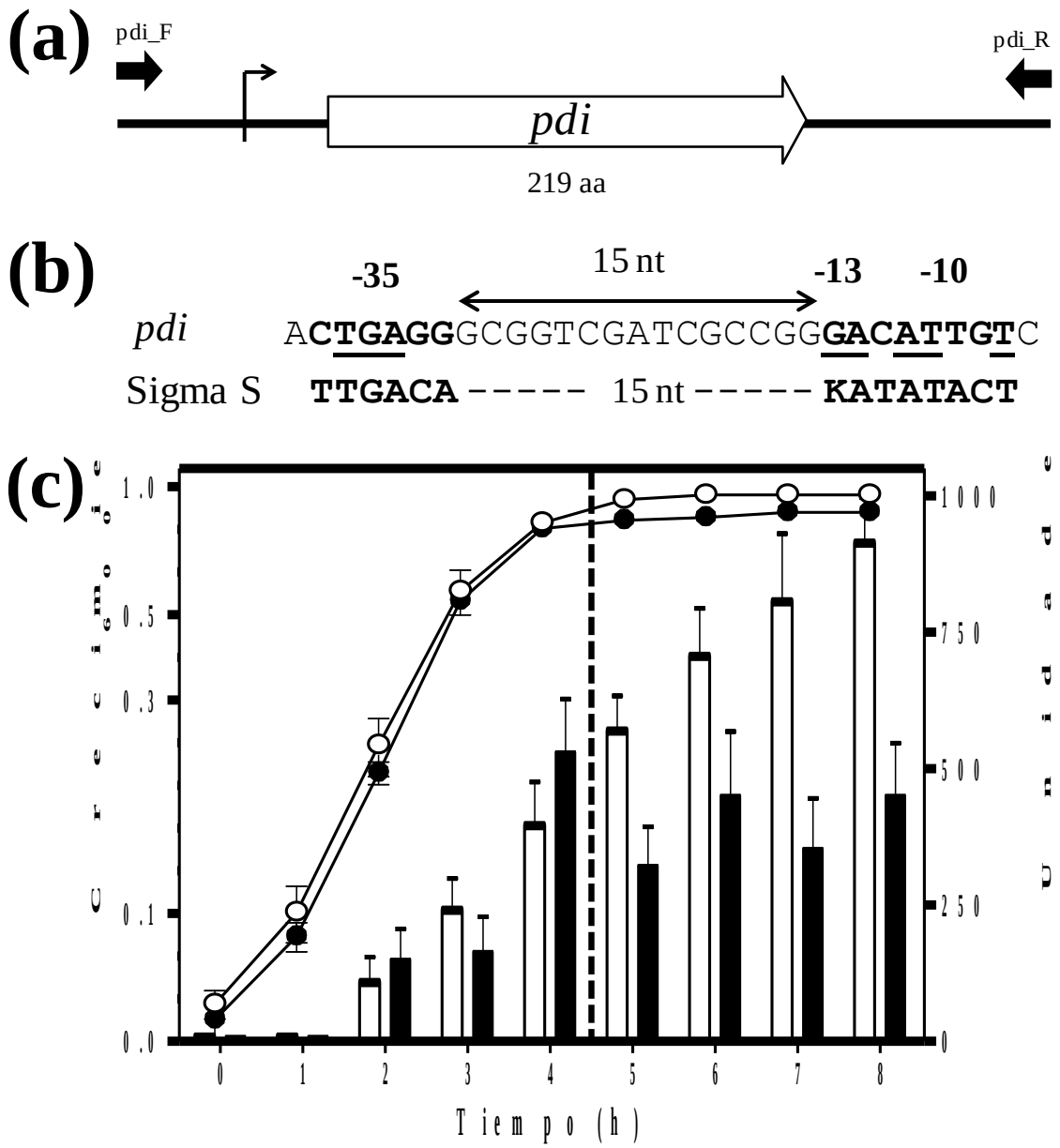
Leyendas de figuras

Figura 1. Análisis transcripcional del gen *pdi*. (a) La región codificante del gen *pdi* se muestra con una flecha larga abierta indicando la dirección de la transcripción; el tamaño del polipéptido predicho (aa) se indica en la parte inferior. La localización del posible promotor se indica por (↱). Las flechas negras muestran la localización de los oligonucleótidos, descritos en el texto. **(b)** Alineamiento del posible promotor de *pdi* y el consenso del promotor dependiente del factor Sigma S. Las secuencias consenso se indican en negritas y los nucleótidos conservados están subrayados. K: nucleótidos G o T. **(c)** Crecimiento de cultivos de (○) *P. aeruginosa* PAO1 (pLP_pdi) y la mutante afectada en el gen *rpoS* (●) PW7152 (pLP_pdi); la actividad de β-galactosidasa de los cultivos se muestra en barras blancas y negras, respectivamente. La línea punteada indica el punto de corte que distingue las fases exponencial y estacionaria del crecimiento bacteriano. Se muestran las barras de error estándar de la media de tres ensayos independientes.

Figura 2. Alineamiento múltiple de los homólogos Pdi. Se muestra la secuencia de la proteína Pdi del plásmido pUM505; las secuencias de las proteínas codificadas en el cromosoma, indicando el organismo (Paer, *Pseudomonas aeruginosa*) seguido del número de acceso. Las regiones con recuadros corresponden a los residuos idénticos. La posición del sitio catalítico redox $C_{66}XXC_{69}$ y el motivo cis-prolina (P_{184}), se indican con asteriscos y un círculo negro, respectivamente; ambos son motivos característicos de las proteínas pertenecientes a la superfamilia de las tiorredoxinas (Martin 1995). Se indica con cuadros negros el par de residuos de cisteína (C_{111} y

C_{157}) y en rojo el residuo de treonina (T_{183}) conservados en las proteínas de la subclase α-DsbA (Kurz *et al*. 2009). Los alineamientos se calcularon con el algoritmo CLUSTALW y el software BioEdit.

Figura 3. Posible estructura terciaria de la proteína Pdi de pUM505. Se indican los elementos estructurales hélices alfa y láminas beta. Las regiones que corresponden al dominio tiorredoxina (Trx) y el dominio hélice alfa se enmarcan en negro y amarillo, respectivamente; ambas regiones características de las proteínas DsbA (Martin 1995). Se muestran las cisteínas catalíticas (C_{66} y C_{69}) y las adicionales (C_{111} y C_{157}). El amino- y carboxilo terminal de la proteína se indican con N y C, respectivamente. El modelo se construyó utilizando el programa Swiss Model empleando como molde la estructura de la proteína cristalizada DsbA2 de *P. aeruginosa* (4n30.1.A).



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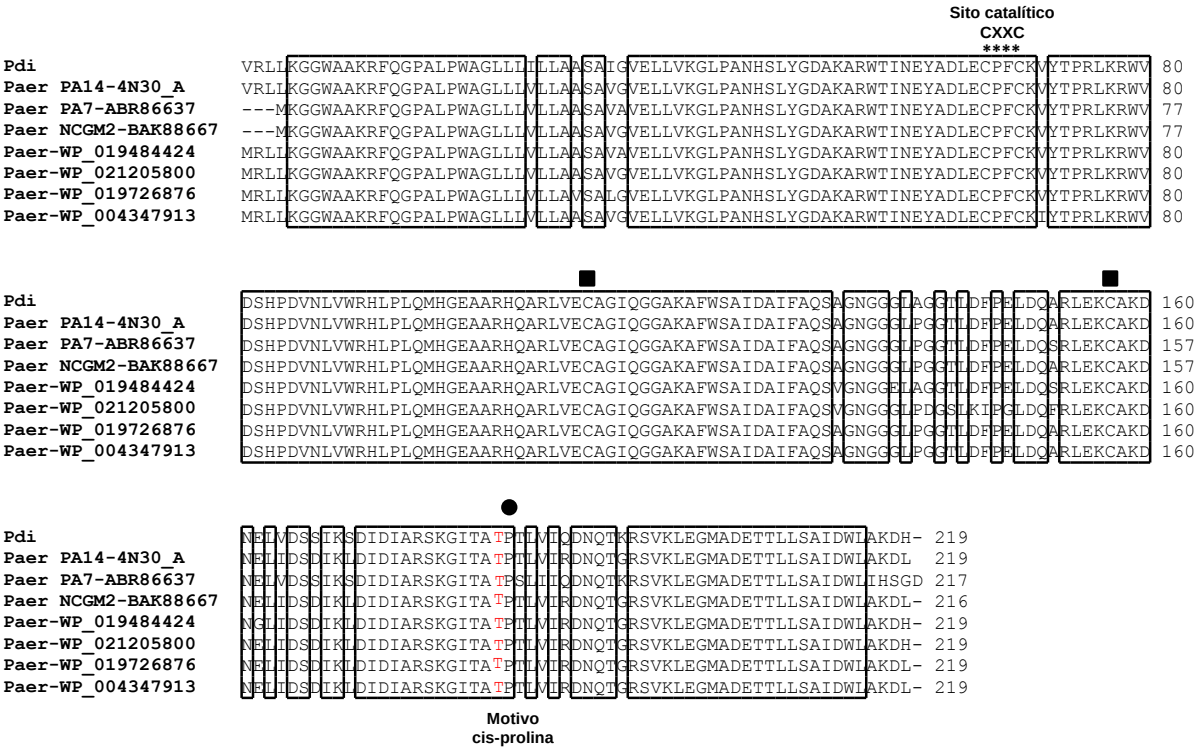
334 Figura 1.


Sito catalítico
CXXC
****
Pdi VRLLKGGWAAKRFQGPALPWAGLLLVLLAASAIGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 80
Paer PA14-4N30_A VRLLKGGWAAKRFQGPALPWAGLLLVLLAASAVGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 80
Paer PA7-ABR86637 ---MKGGWAAKRFQGPALPWAGLLLVLLAASAVAVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 77
Paer NCGM2-BAK88667 ---MKGGWAAKRFQGPALPWAGLLLVLLAASAVGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 77
Paer-WP_019484424 MRLLKGGWAAKRFQGPALPWAGLLLVLLAASAVAVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 80
Paer-WP_021205800 MRLLKGGWAAKRFQGPALPWAGLLLVLLAASAVGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 80
Paer-WP_019726876 MRLLKGGWAAKRFQGPALPWAGLLLVLLAVSALGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKVYTPRLKRWV 80
Paer-WP_004347913 MRLLKGGWAAKRFQGPALPWAGLLLVLLAASAVGVELLVKGLPANHSLYGDAKARWTINEYADLECPFCKIYTPRLKRWV 80
Pdi DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLAGGTLDFPELDQARLEKCAKD 160
Paer PA14-4N30_A DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLPGGTLDFPELDQARLEKCAKD 160
Paer PA7-ABR86637 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLPGGTLDFPELDQSRLEKCAKD 157
Paer NCGM2-BAK88667 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLPGGTLDFPELDQARLEKCAKD 157
Paer-WP_019484424 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSVGNGGGLAGGTLDFPELDQSRLEKCAKD 160
Paer-WP_021205800 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSVGNGGGLPDGSLKIPGLDQFRLEKCAKD 160
Paer-WP_019726876 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLPGGTLDFPELDQARLEKCAKD 160
Paer-WP_004347913 DSHPDVNLVWRHLPLQMHGEAARHQARLVECAGIQGGAKAFWSAIDAIFAQSAGNGGGLPGGTLDFPELDQARLEKCAKD 160
Pdi NELVDSSIKSDIDIARSKGITTPTLVLQDNQTKRSVKLEGMADETTLLSAIDWLAKDH- 219
Paer PA14-4N30_A NELIDSDIKLDIDIARSKGITATPTLVIRDNQTGRSVKLEGMADETTLLSAIDWLAKDL 219
Paer PA7-ABR86637 NELVDSSIKSDIDIARSKGITATPSLIIQDNQTKRSVKLEGMADETTLLSAIDWLIHSGD 217
Paer NCGM2-BAK88667 NELIDSDIKLDIDIARSKGITATPTLVIRDNQTGRSVKLEGMADETTLLSAIDWLAKDL- 216
Paer-WP_019484424 NGLIDSDIKLDIDIARSKGITATPTLVIRDNQTGRSVKLEGMADETTLLSAIDWLAKDH- 219
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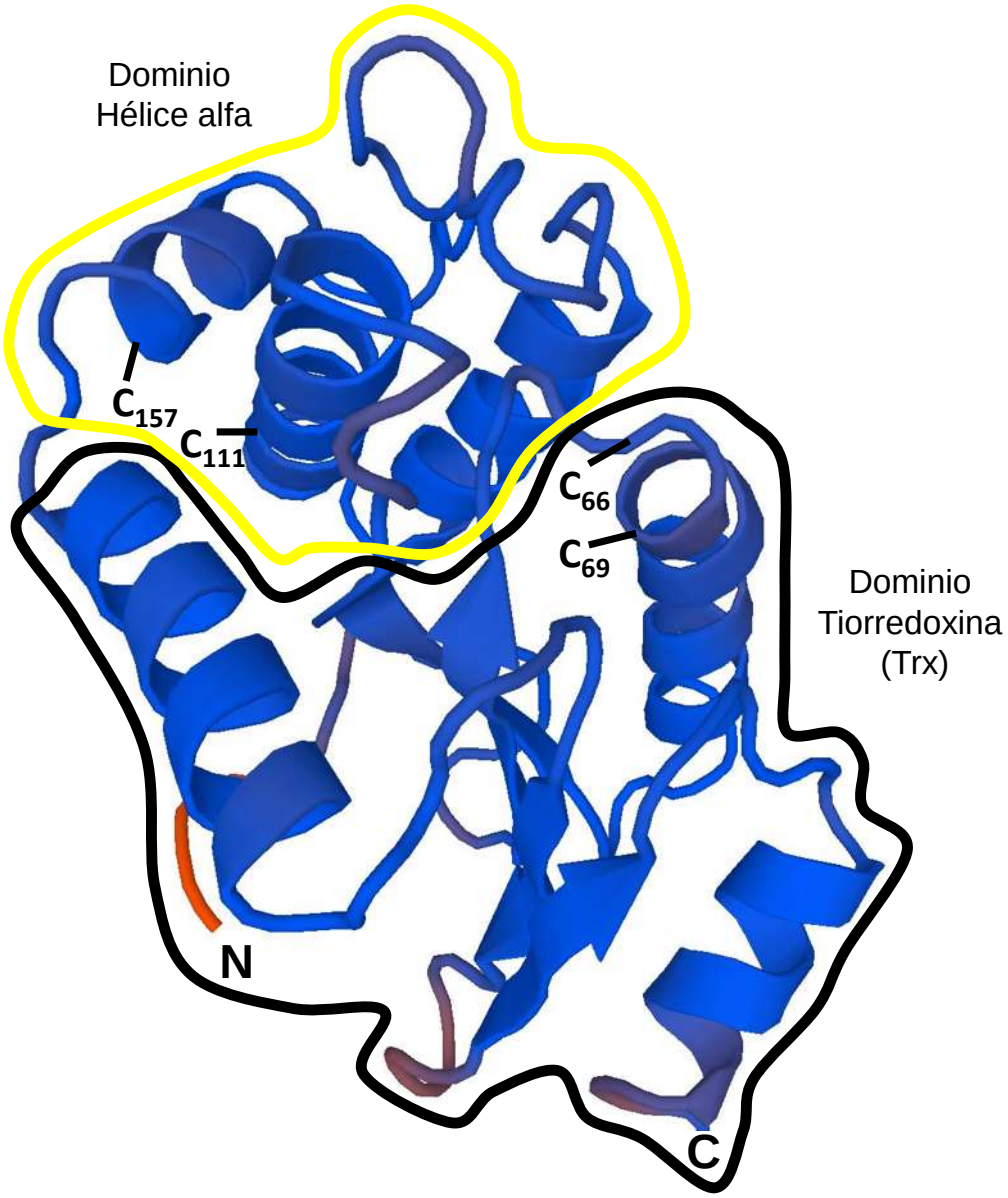
Paer-WP_004347913 NELIDSDIKLDIDIARSKGITATPTLVIRDNQTGRSVKLEGMADETTLLSAIDWLAKDL- 219
Motivo
cis-prolina

Figura 2.



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339 Figura 3.

VII. DISCUSIÓN GENERAL

En la naturaleza, los plásmidos incrementan la diversidad genética y promueven la adaptación bacteriana por la transferencia horizontal de genes, proceso que introduce información genética no parental dentro de una célula. El plásmido conjugativo pUM505 se aisló de una cepa clínica de *P. aeruginosa* obtenida de un paciente hospitalizado, y se determinó que es capaz de conferir resistencia a cromato y mercurio (Cervantes *et al.* 1990). El primer mecanismo descrito de resistencia bacteriana a cromato es el conferido por el gen *chrA* codificado en pUM505 (Alvarez *et al.* 1999) y es el mejor caracterizado en bacterias (Ramírez-Díaz *et al.* 2008).

El plásmido pUM505 presenta dos regiones bien definidas, la primera corresponde a una isla genómica (IG), que muestra homología con genes presentes en la isla de patogenicidad PAPI-1 de *P. aeruginosa* PA14. Esta isla posee genes que codifican proteínas implicadas en la replicación y transferencia del plásmido, así como proteínas probablemente involucradas en la virulencia y metabolismo de proteínas. Esta PAI pertenece a la familia de IGs denominada pKLC102 y relacionadas, que predominan en beta- y gammaproteobacterias (Klockgether *et al*. 2007). Los miembros de esta familia tienen un origen híbrido plásmido-fago que consiste de dos partes: i) un grupo de genes conservados involucrados en la propagación, replicación y partición; y ii) un grupo de genes variables implicados en propiedades adaptativas (Klockgether *et al*. 2004, 2007; Würdemann y Tümmler 2007). El análisis de las diversas IGs de la familia pKLC102 ha permitido deducir la historia evolutiva de estas IGs, que podrían proceder de un plásmido integrativo ancestral (Klockgether *et al*. 2004; Kulasekara *et al.* 2006; Battle *et al.*

2008). La segunda región del plásmido pUM505 corresponde a una isla de resistencia a metales pesados, con genes que codifican proteínas implicadas en la resistencia a cromato y mercurio (Fig. 1). Esta región también contiene varias características frecuentemente asociadas con IGs, incluyendo la presencia de repetidos invertidos, genes implicados en la movilidad (que codifican integrasas, resolvasas y transposasas) así como elementos de inserción.

El plásmido pUM505 también contiene el operón *umuD*/*umuC* (*umuDC*) que codifican proteínas similares a la DNA polimerasa V propensa a error. Los genes *umuDC* forman parte de la respuesta SOS, que se describió por primera vez en *E. coli* (Walker 1984). El gen *umuC* aparentemente está truncado y su producto probablemente no es funcional. El gen *umuD* se renombró *umuDpR* (por *umuD* *p*lasmid *R*egulator), éste posee una caja SOS traslapada con el probable promotor reconocido por el factor Sigma-70. La caja SOS de *umuDpR* es similar a la región reguladora de los genes *umuDC de E. coli* (Kitagawa *et al.,* 1985) y de sus homólogos *rulAB* presentes en el plásmido pWW0 de *P. putida* (Tark *et al.,* 2005); mediante fusiones transcripcionales se demostró en este trabajo que el promotor del gen *umuDpR* es activado por Mitomicina C (MMC), agente generador de daño al DNA. La secuencia predicha de la proteína UmuDpR mostró 23% de identidad con LexA de *P. aeruginosa*, represor de la respuesta SOS, así como al regulador UmuDAb de *Acinetobacter baylyi* y *A. baumannii*. El gen *umuDp*R causó una sensibilidad incrementada a MMC cuando se transfirió a la cepa de *P. aeruginosa* PAO1. Como se esperaba, la mutante PW6037 derivada de PAO1 afectada en el gen *lexA* mostró resistencia a MMC; sin embargo, al transferir el gen *umuDpR* a la

mutante PW6037, el nivel de resistencia a MMC disminuyó. Estos datos sugieren que UmuDpR reprime la expresión de genes SOS de manera similar que LexA. Se determinó, mediante ensayos de qRT-PCR, que la expresión de los genes *lexA*, *imuA* y *recA*, genes SOS de PAO1, incrementó de 3.4 a 5.3 veces en la mutante *lexA*$^{-}$, con respecto a la expresión transcripcional de dichos genes en la cepa *lexA*$^{+}$, pero ésta disminuyo significativamente en la transformante *lexA*$^{-}$/*umuDp*R. Estos resultados confirmaron que la proteína UmuDpR es un represor de los genes SOS de *P. aeruginosa* controlados por LexA. Sin embargo, mediante ensayos de cambio de movilidad electroforética, se encontró que UmuDpR no se une a la región reguladora de los genes SOS, sugiriendo un mecanismo indirecto de regulación. Probablemente UmuDpR requiere cofactor(es) adicional(es) para completar su función de regulación, como previamente se reportó para la proteína homóloga UmuDAb codificada en el cromosoma de *A. baylyi* (Hare *et al.* 2012).

Por lo tanto, el producto del gen *umuD* del plásmido pUM505 es capaz de reprimir la expresión de genes SOS de *P. aeruginosa*, lo cual sugiere que la posesión de este factor anti-SOS es una ventaja evolutiva para pUM505.

VIII. BIBLIOGRAFÍA COMPLEMENTARIA

1. Acosta-Navarrete, Y. M., León-Márquez, Y. L., Salinas-Herrera, K., Jácome-Galarza, I. E., Meza-Carmen, V., Ramírez-Díaz, M. I. y Cervantes, C. (2014) Expression of the six chromate ion transporter homologues of *Burkholderia xenovorans* LB400. *Microbiology* **160**, 287-295.
2. Alvarez, A.H., Moreno-Sánchez, R. y Cervantes, C. (1999). Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. *J. Bacteriol*. **181**, 7398-7400.
3. Barkay, T., Miller, S.M. y Summers, A.O. (2003) Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol. Rev.* **27**; 355-384.
4. Battle SE, Meyer, F., Rello, J., Kung, V.L. y Hauser, A.R. (2008) The hybrid pathogenicity island PAGI-5 contributes to the highly virulent phenotype of a *Pseudomonas aeruginosa* isolate in mammals. *J. Bacteriol.* **190**, 7130-7140.
5. Battle, S.E., Rello, J. y Hauser, A.R. (2009) Genomic islands of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **290**, 70-78.
6. Branco, R., Chung, A.P., Johnston, T., Gurel, V., Morais, P. y Zhitkovich, A. (2008) The chromate-inducible *chrBACF* operon from the transposable element TnOtChr confers resistance to chromium(VI) and superoxide. *J. Bacteriol.* **190**, 6996-7003.
7. Cervantes, C., Ohtake, H., Chu, L., Misra, T.K. y Silver, S. (1990) Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. *J. Bacteriol.* **172**, 287-291.
8. Chivers, P.T., Prehoda, K.E. y Raines, R.T. (1997) The CXXC motif: a rheostat in the active site. *Biochemistry* **36**, 4061-4066.

9. Darmon, E. y Leach, D.R.F. (2014) Bacterial Genome Instability. *Microbiol. Mol. Biol. Rev.* **78**, 1-39.

10. Dobrindt, U., Hochhut, B., Hentschel, U. y Hacker J. (2004) Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol*. **2**, 414-424.

11. Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J.P., Fleiszig, S.M., Wu, C., Mende-Mueller, L., y Frank, D.W. (1997) ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol. Microbiol.* **25**, 547-557.

12. Frost, L.S., Leplae, R., Summers, A.O. y Toussaint, A. (2005) Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol*. **3**, 722-732.

13. Frost, L.S. y Koraimann, G. (2010) Regulation of bacterial conjugation: balancing opportunity with adversity. *Future Microbiol.* **5**, 1057-1071.

14. Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R. y Goebel, W. (1990) Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extraintestinal *Escherichia coli* isolates. *Microb. Pathog.* **8**, 213-225.

15. Hauser, A.R., Kang, P.J. y Engel J.N. (1998) PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. *Mol. Microbiol.* **27**, 807-818.

16. He, J., Baldini, R.L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N.T. *et al.* (2004) The broad host range pathogen *Pseudomonas aeruginosa* strain

PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl. Acad. Sci. USA* **101**, 2530-2535.

17. Hughes, V.M. y Datta, N. (1983) Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**, 725-726.
18. Juhas, M., van der Meer, J.R., Gaillard, M., Harding, R.M., Hood, D.W. y Crook, D.W. (2009) Genomic islands: tools of bacterial horizontal gene transfer and evolution. *FEMS Microbiol. Rev.* **33**, 376-93.
19. Juhnke, S., Peitzsch, N., Hübener, N., Grosse, C. y Nies, D.H. (2002) New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34. *Arch. Microbiol.* **179**, 15-25.
20. Kitagawa, Y., Akaboshi E, Shinagawa, H., Horii, T., Ogawa, H. y Kato, T. (1985). Structural analysis of the *umu* operon required for inducible mutagenesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **82**, 4336-4340.
21. Kivisaar, M. (2010). Mechanisms of stationary-phase mutagenesis in bacteria: mutational processes in pseudomonads. *FEMS Microbiol. Lett.* **312**, 1-14.
22. Klockgether, J., Reva, O., Larbig, K. y Tümmler, B. (2004) Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. *J. Bacteriol.* **186**, 518-534.
23. Klockgether, J., Reva, O., Larbig, K. y Tümmler, B. (2007) Diversity of the abundant pKLC102/PAGI-2 family of genomic islands in *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**, 2443-2459.
24. Kulasekara, B.R., Kulasekara, H.D., Wolfgang, M.C., Stevens, L., Frank, D.W. y Lory S. (2006) Acquisition and evolution of the *exoU* locus in *Pseudomonas aeruginosa. J. Bacteriol.* **188**, 4037-4050.

25. Kung, V.L., Ozer, E.A. y Hauser, A.R. (2010) The Accessory genome of *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev*. **74**, 621-641.

26. Levin, B.R. y Bergstrom, C.T. (2000) Bacteria are different: observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. *Proc. Natl. Acad. Sci. USA* **97**, 6981-6985.

27. Liang, X., Pham, X.Q., Olson, M.V. y Lory, S. (2001) Identification of a genomic island present in the majority of pathogenic isolates of *Pseudomonas aeruginosa*. *J. Bacteriol*. **183**, 843-853.

28. Migula N. (1894) Arbeiten aus dem Bakteriologischen Institut der Technischen Hochschule zu Karlsruhe **1**, 235-238.

29. Molbak, L., Tett, A., Ussery, D.W., Wall, K., Turner, S., Bailey, M. y Field, D. (2003) The plasmid genome database. *Microbiology* **149**, 3043-3045.

30. Novick, R.P. (1969) Extrachromosomal inheritance in bacteria. *Bacteriol. Rev.* **33**, 210-263.

31. Özen A.I. y Ussery D.W. (2012) Defining the *Pseudomonas* genus: where do we draw the line with *Azotobacter*? *Microb. Ecol.* **63**, 239-248.

32. Nohmi, T. (2006). Environmental stress and lesion-bypass DNA polymerases. *Annu. Rev. Microbiol.* **60**, 231-253.

33. Patel, M., Jiang, Q., Woodgate, R., Cox, M.M. y Goodman, M.F. (2010). A new model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. *Crit. Rev. Biochem. Mol. Biol.* **45**, 171-184.

34. Peix, A., Ramírez-Bahena, M.H. y Velázquez, E. (2009) Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infect. Genet. Evol*. **9**, 1132-1147.

35. Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front. Microbiol.* **2**, 65.

36. Ramírez-Díaz, M.I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J. y Cervantes, C. (2008). Mechanisms of bacterial resistance to chromium compounds. *Biometals* **21**, 321-332.

37. Sato, H., Frank, D.W., Hillard, C.J., Feix, J.B., Pankhaniya, R.R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R.M., Wiener-Kronish, J. y Sawa, T. (2003) The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. *EMBO J.* **22**, 2959-2969.

38. Shintani, M., Sanchez, Z.K. y Kimbara, K. (2015) Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. *Front. Microbiol*. **6**, 242.

39. Slater, F.R., Bailey, M.J., Tett, A.J. y Turner, S.L. (2008) Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiol. Ecol.* **66**, 3-13.

40. Smillie, C., Garcillan-Barcia, M.P., Francia, M.V., Rocha, E.P.C. y de la Cruz, F. (2010) Mobility of plasmids. *Microbiol. Mol. Biol. Rev*. **74**, 434-452.

41. Sota, M. y Top, E. (2008) “Horizontal gene transfer mediated by plasmids,” in Plasmids: Current Research and Future Trends, ed G. Lipps (Norfolk,VA: Caister Academic Press; Horizon Scientific Press), 111-181.

42. Strateva, T. y Mitov, I. (2011) Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections. *Ann. Microbiol.* **61**, 717-732.

43. Tark, M., Tover, A., Tarassova, K., Tegova, R., Kivi, G., Horak, R. y Kivisaar, M. (2005). A DNA polymerase V homologue encoded by TOL plasmid pWW0 confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress. *J. Bacteriol.* **187**, 5203-5213.

44. Walker, G.C. (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. *Microbiol. Rev.* **48**, 60-93.

45. Watanabe, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bacteriol. Rev*. **27**, 87-115.

46. Würdemann, D. y Tümmler, B. (2007) *In silico* comparison of pKLC102-like genomic islands of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **275**, 244-249.