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**DETERMINACIÓN DEL MECANISMO DE
REGULACIÓN DEL SISTEMA TOXINA-ANTITOXINA
DEL PLÁSMIDO pUM505**

TESIS

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I. RESUMEN

Los sistemas Toxina-Antitoxina (TA) son elementos genéticos pequeños compuestos de un gen toxina y su cognado antitoxina, los cuales son importantes para la estabilización de plásmidos (codificados en plásmidos) y en la virulencia bacteriana (codificados en cromosomas). En los sistemas TA, las toxinas son proteínas que inhiben el crecimiento o generan muerte celular, mientras que las antitoxinas regulan la actividad de la toxina, ya sea por su unión con la toxina o por la inhibición de la expresión del gen que la codifica. En nuestro grupo de trabajo se determinó que los genes *pumA* y *pumB* del plásmido pUM505, aislado de una cepa clínica de *Pseudomonas aeruginosa*, forman un operón que codifica un sistema TA que participa en la estabilidad de plásmidos. Además, la toxina PumA incrementa la virulencia de *P. aeruginosa* PAO1 en ratón, efecto que es neutralizado por la presencia del gen de la antitoxina *pumB*, sugiriendo que PumA puede actuar como un factor de virulencia y que PumB puede regular la función de la toxina. *P. aeruginosa* es un patógeno humano resistente a antibióticos que produce factores de virulencia modulados por Quorum Sensing (QS). La proteína PumB mostró identidad con antitoxinas tipo HTH de unión a DNA sugiriendo que la proteína PumB puede regular al sistema TA al unirse a la región promotora del operón *pumAB*. Por lo anterior, resultó de interés caracterizar la forma de regulación del sistema TA PumAB de pUM505. En este trabajo se determinó que la expresión de las proteínas PumA y PumB en *Escherichia coli* BL21 no generó inhibición del crecimiento de la bacteria, sugiriendo que la co-expresión de ambas proteínas es necesaria para que PumB pueda neutralizar los efectos tóxicos de PumA. Transformantes de *P. aeruginosa* PAO1 con el gen *pumA* incrementaron la muerte de *Caenorhabditis elegans*, efecto que fue neutralizado por la proteína PumB. Este mismo efecto de muerte del nematodo fue observado al emplear el sobrenadante del cultivo de *P. aeruginosa* PAO1 (pUC_pumA), sugiriendo que la toxina es secretada al exterior de la célula. Además, la proteína recombinante His-PumA purificada generó la muerte de *C. elegans*, indicando que la proteína PumA actúa como una toxina, efecto que fue neutralizado por la previa incubación *in vitro* de PumA con PumB, sugiriendo que PumB regula la actividad de la toxina a través de su unión con PumA. El análisis *in silico* de los probables elementos regulatorios del operón *pumAB* mostró la presencia de dos promotores internos para el gen *pumB*, además del promotor σ^{70} previamente reportado para el operón. El análisis de expresión de los genes *pumA* y *pumB* mostró que tanto en *P. aeruginosa* PAO1 (pUM505) como en *E. coli* BL21 (pTrc2A_pumAB), los niveles de RNAm de *pumB* se encuentran incrementados respecto al RNAm de *pumA*, sugiriendo que los genes del operón *pumAB* se expresan de

manera diferencial o que el RNAm de *pumA* es degradado. Además, se determinó que *pumB* puede ser expresado individualmente y que a diferencia del gen *pumA* sus niveles de RNAm disminuyen en presencia de un agente oxidante como el peróxido de hidrogeno, sugiriendo la regulación negativa de *pumB* bajo condiciones de estrés. Mediante ensayos de cambio de movilidad electroforética se determinó que PumB se une a una secuencia palindrómica cercana al probable promotor σ^{70} del operón *pumAB*, pero no es capaz de unirse a la región de DNA donde se localizan los promotores de *pumB*, sugiriendo que la antitoxina regula la expresión del operón *pumAB*. Por otro lado, ensayos *in vitro* de la cuantificación del RNAm del gen *pumA* remanente, después de su incubación con la proteína PumB purificada, mostraron que el nivel del RNAm de *pumA* disminuyó al ser incubado en presencia de PumB, efecto que fue mayor al incrementar la concentración de la proteína PumB. Esto sugirió que PumB degrada el RNAm del gen *pumA*. La capacidad de la proteína PumB de degradar el RNAm de *pumA* se inhibió al incubarla previamente con inhibidores de ribonucleasas, sugiriendo que PumB posee actividad de ribonucleasa sobre el RNAm de *pumA*. El gen *pumA* fue transferido a la cepa de *P. aeruginosa lasI/rhlI*, una mutante en los sistemas LasI y RhlI del QS, para analizar el efecto de la virulencia de las transformantes. La transferencia de *pumA* no aumentó la virulencia de la bacteria en lechuga y en *C. elegans*, aunado a que los niveles de RNAm de *pumA* disminuyeron drásticamente en comparación con la cepa de *P. aeruginosa* (pUC_*pumA*), sugiriendo que la virulencia conferida por PumA es dependiente de QS. El crecimiento de *P. aeruginosa lasI/rhlI* (pUC_*pumA*) en medio de cultivo suplementado con los autoinductores C4-AHL y 3-oxo-C12-AHL de QS, incrementó los niveles de RNAm de *pumA* y restauró la virulencia de la bacteria, indicando que ambos autoinductores complementan la mutación y regulan positivamente los efectos tóxicos de PumA, sugiriendo que el QS regula la virulencia de la bacteria conferida por la toxina PumA. Estos resultados en conjunto sugieren que el sistema TA PumAB posee múltiples mecanismos para regular su expresión, así como los niveles de RNAm de la toxina y la virulencia producida por *pumA* es regulada por QS.

Palabras Claves: Sistema Toxina-Antitoxina, estabilidad plásmidica, *Pseudomonas aeruginosa*, regulación genética, plásmidos, Quorum sensing.

II. ABSTRACT

Toxin-antitoxin (TA) systems are small genetic elements made up of a toxin gene and its antitoxin cognate, which are important for the stabilization of plasmids (encoded in plasmids) and in bacterial virulence (encoded in chromosomes). In TA systems, toxins are proteins that inhibit growth or cause cell death, while antitoxins regulate the activity of the toxin, either by binding to the toxin or by inhibiting the expression of the gene that encodes for it. In our working group, it was determined that the *pumA* and *pumB* genes of the plasmid pUM505, isolated from a clinical strain of *Pseudomonas aeruginosa*, form an operon that encodes a TA system that participates in the stability of plasmids. Furthermore, the PumA toxin increases the virulence of *P. aeruginosa* PAO1 in mice, an effect that is neutralized by the presence of the *pumB* antitoxin gene, suggesting that PumA may act as a virulence factor and that PumB may regulate toxin function. *P. aeruginosa* is an antibiotic-resistant human pathogen that produces virulence factors modulated by Quorum Sensing (QS). The PumB protein showed identity with DNA binding HTH antitoxins suggesting that the PumB protein may regulate the TA system by binding to the promoter region of the *pumAB* operon. Therefore, it was interesting to characterize the form of regulation of the TA PumAB system of pUM505. In this work, it was determined that the expression of the PumA and PumB proteins in *Escherichia coli* BL21 did not inhibited the bacterium growth, suggesting that the co-expression of both proteins is necessary for PumB to neutralize the toxic effects of PumA. Transformants of *P. aeruginosa* PAO1 with the *pumA* gene increased *Caenorhabditis elegans* death, an effect that was neutralized by the PumB protein. This same nematode killing effect was observed when the supernatant from the *P. aeruginosa* PAO1 (pUC_pumA) culture was used, suggesting that the toxin is secreted of the cell. Furthermore, the purified His-PumA recombinant protein caused the death of *C. elegans*, indicating that the PumA protein acts as a toxin, an effect that was neutralized by the previous *in vitro* incubation of PumA with PumB, suggesting that PumB regulates the activity of the toxin through its binding with PumA. *In silico* analysis of the probable regulatory elements of the *pumAB* operon showed the presence of two internal promoters for the *pumB* gene, in addition to the previously reported σ^{70} promoter for the operon. Expression analysis of the *pumA* and *pumB* genes showed that in both *P. aeruginosa* PAO1 (pUM505) and *E. coli* BL21 (pTrc2A_pumAB), the levels of *pumB* mRNA are increased respect to the *pumA* mRNA, suggesting that the genes of the *pumAB* operon are differentially expressed or that the *pumA* mRNA is degraded. Furthermore, it was determined that *pumB* can be expressed individually and that, unlike the *pumA* gene, its mRNA levels decrease in the presence of an

oxidizing agent such as hydrogen peroxide, suggesting the negative regulation of *pumB* under stress conditions. Electrophoretic mobility shift assays determined that PumB binds to a palindromic sequence close to the probable σ^{70} promoter of the *pumAB* operon, but is unable to bind to the region of DNA where the *pumB* promoters are located, suggesting that the antitoxin regulates *pumAB* operon expression. On the other hand, *in vitro* mRNA quantification assays of the remaining *pumA* gene, after its incubation with the purified PumB protein, showed that the level of *pumA* mRNA decreased when incubated in the presence of PumB, an effect that was greater with increasing the PumB protein concentration. This suggested that PumB degrades the *pumA* gene mRNA. The ability of the PumB protein to degrade *pumA* mRNA was inhibited by previously incubating it with ribonuclease inhibitors, suggesting that PumB possesses ribonuclease activity on *pumA* mRNA. The *pumA* gene was transferred to the *P. aeruginosa* lasI/rhlI strain, a mutant in the LasI and RhlI systems of the QS, to analyze the virulence effect of the transformants. Transfer of *pumA* did not increase the virulence of the bacteria in lettuce and *C. elegans*, in addition to that the levels of *pumA* mRNA decreased drastically compared to the *P. aeruginosa* (pUC_ *pumA*) strain, suggesting that the virulence conferred by PumA is dependent on QS. Growth of *P. aeruginosa* lasI/rhlI (pUC_ *pumA*) in culture medium supplemented with autoinductors C4-AHL and 3-oxo-C12-AHL of QS, increased *pumA* mRNA levels and restored the bacterium virulence, indicating that both autoinducers complement the mutation and positively regulate the toxic effects of PumA, and suggesting that QS regulates the virulence of the bacterium conferred by the PumA toxin. These results together suggest that the TA PumAB system has multiple mechanisms to regulate its expression, as well as the levels of toxin mRNA and the virulence produced by *pumA* is regulated by QS.

Key Words: Toxin-Antitoxin System, plasmid stability, *Pseudomonas aeruginosa*, genetic regulation, plasmids, Quorum sensing.

III. INTRODUCCIÓN

1. Sistemas toxina-antitoxina (TA)

Los sistemas TA son elementos genéticos pequeños compuestos de un gen que codifica a una toxina y un gen que codifica a una antitoxina. Se encuentran en elementos genéticos móviles bacterianos y en cromosomas bacterianos, actualmente se agrupan en los seis tipos de sistemas TA (Leplae *et al.*, 2011; Page and Peti, 2016). Los sistemas TA son codificados por genes que forman un operón, el espaciador rara vez se extiende más allá de 30 pares de bases (pb), y un pequeño solapamiento (1 a 20 pb en general) es la estructura más común. El orden de los dos genes también está bien conservado, con la antitoxina río arriba (**Figura 1**), esta disposición contribuye a una alta proporción de antitoxina a toxina y asegura que la toxina se inactive en condiciones sin estrés (revisado en Sofos *et al.*, 2015). Aunque hay una excepción, en el *loci higBA* el gen de la toxina está localizado río arriba del de la antitoxina (Tian *et al.*, 1996). En estos casos, tales diferencias en el orden de los genes podrían ejercer un efecto sutil sobre la proporción de toxina a antitoxina en la célula, que a su vez regula la función de la toxina (Yamaguchi *et al.*, 2011). Por lo general, la transcripción de operones TA está regulada por la unión de la antitoxina o por un complejo formado por proteínas de toxina y antitoxina (Brzozowska and Zielenkiewicz, 2013). Todos los genes TA analizados son pequeños, su tamaño puede variar entre 120pb y 630 pb (Sevin and Barloy- Hubler, 2007). Según Pandey y Gerdes (2005), las antitoxinas presentan de 41 a 206 aminoácidos (aa) de longitud y las toxinas de 31 a 204 aa; también en este caso hay una excepción: la toxina del sistema HipBA es de 440 aa. Las antitoxinas son generalmente más cortas que sus toxinas asociadas (Korch and Hill, 2006).

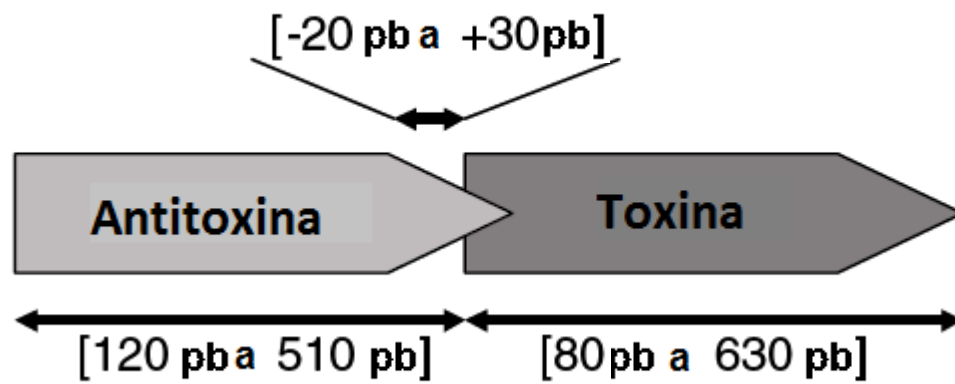


Figura 1. Contexto genético general de loci TA. Se muestra la organización típica de loci TA con tamaños y perfiles de distancia (Adaptada de Sevin and Barloy-Hubler, 2007).

Las toxinas de los sistemas TA son proteínas, mientras que las antitoxinas son proteínas o RNAs pequeños (sRNA) (Unterholzner *et al.*, 2013). Las toxinas de los sistemas TA se consideran moléculas dañinas para las células debido a sus efectos inhibitorios sobre el crecimiento celular, lo que lleva a la estasis de crecimiento en condiciones estresantes (Park *et al.*, 2013). Las antitoxinas son más lábiles y se degradan más fácilmente en condiciones de estrés que las toxinas, lo que permite que las toxinas ejerzan sus efectos tóxicos (Yamaguchi *et al.*, 2011).

El primer sistema TA, *ccdA/ccdB*, fue descrito hace 30 años como un módulo de mejora de la estabilidad del plásmido F de *Escherichia coli*. Los sistemas TA codificados por plásmidos también se llaman módulos de adicción (Yarmolinsky, 1995), ya que esta propiedad hace que la célula sea “adicta” o requiera la producción de antitoxina y por lo tanto a los genes TA. La amplia difusión de los sistemas TA en los genomas de bacterias es una consecuencia de su capacidad de moverse por la transferencia horizontal de genes (Pandey and Gerdes, 2005; Koonin and Wolf, 2008). Los sistemas TA han sido descritos también en los cromosomas, especialmente en islas genómicas, donde a menudo se encuentran en varias copias. Estos sistemas codificados cromosómicamente se han asociado con la virulencia de patógenos bacterianos (Hayes and Van Melderren, 2011; Unterholzner *et al.*, 2013; De la Cruz *et al.*, 2013).

2. Clasificación de los sistemas TA

Los sistemas TA se han clasificado en seis tipos diferentes de acuerdo con la naturaleza de las antitoxinas y la composición del sistema TA (Page and Peti, 2016). Los tipos I y III poseen antitoxinas de RNA que inhiben la síntesis de toxinas o

secuestran su toxina, mientras que los tipos II, IV, V y VI consisten en antitoxinas proteicas (revisado en Goeders and Van Melderren, 2014) (**Figura 2**):

❖ Tipo I

Las antitoxinas del Tipo I son RNAs antisentido inestables. En la mayoría de los casos la expresión de la toxina está regulada por apareamiento de bases del RNA de la antitoxina con el RNAm estable de la toxina. Esta interacción ha demostrado prevenir la unión al ribosoma y de este modo detener la traducción del RNAm de la toxina.

❖ Tipo II

Los sistemas TA de tipo II son los más estudiados en la actualidad debido a su abundancia en las bacterias y arqueas. Los genes están organizados en operones con la antitoxina situada río arriba del gen de la toxina, aunque se conocen algunos ejemplos de organización invertida de genes, por ejemplo, *higBA* (Tian *et al.*, 1996), *hicAB* (Jørgensen *et al.*, 2009), *mqsRA* de *E. coli* (Brown *et al.*, 2009) y *brnTA* de *Brucella abortus* (Heaton *et al.*, 2012). Por lo general, la transcripción de genes TA es regulada sólo por la antitoxina o por el complejo formado por ambas proteínas; sin embargo, también se conocen sistemas de tres componentes, donde la tercera proteína juega un papel regulador específico (Smith and Rawlings, 1997; De la Hoz *et al.*, 2000). La presencia de un exceso de la antitoxina respecto a la toxina asegura la formación de un complejo de estas dos proteínas y la inactividad de la toxina. En una situación en la que el nivel celular de la antitoxina disminuye, la toxina ya no puede ser neutralizada.

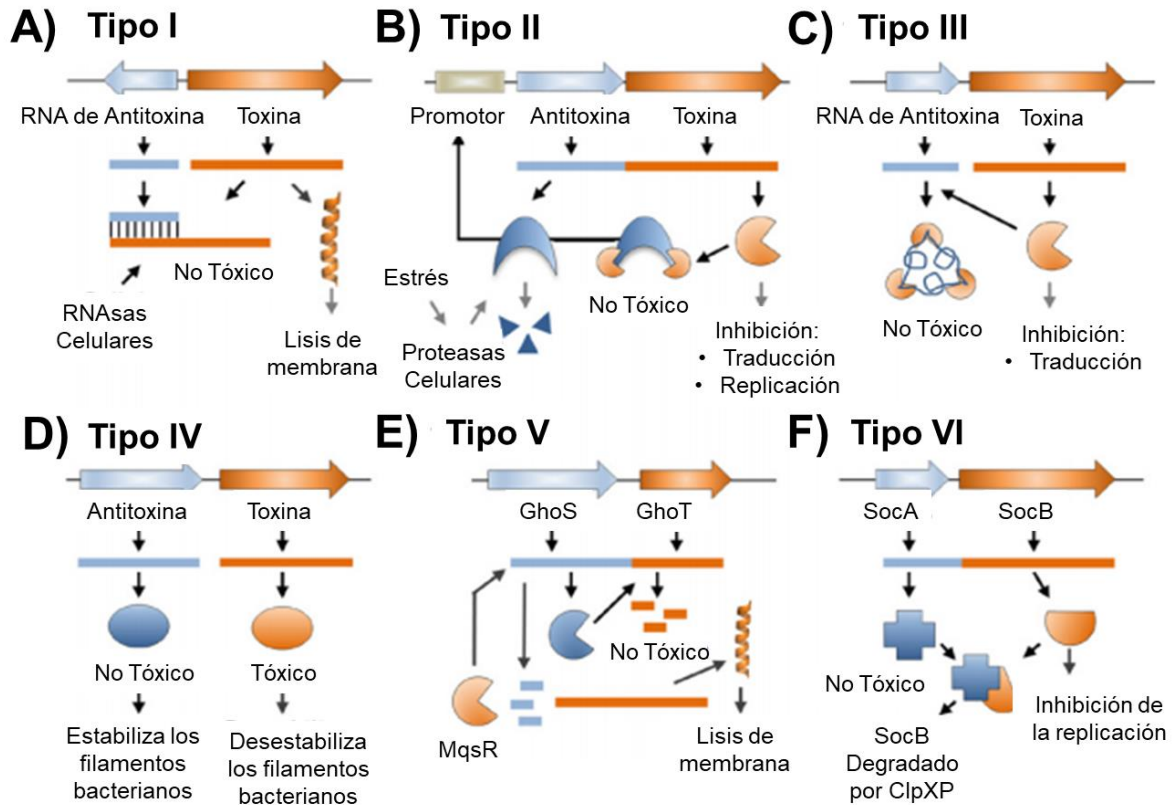


Figura 2. Tipos de Sistemas TA. Las toxinas se muestran en naranja y las antitoxinas en azul. A) Tipo I: la antitoxina RNAs se empareja con el RNAm de la toxina para inhibir la traducción. B) Tipo II: la antitoxina y la toxina son proteínas; en condiciones de crecimiento, la toxina se une a la antitoxina, lo que inhibe su actividad. Tanto la antitoxina como, en la mayoría de los casos, el complejo TA se unen al promotor TA para reprimir la transcripción. En condiciones de estrés, se activan las proteasas celulares como Lon y ClpXP que escinden preferentemente las antitoxinas, liberando las toxinas para inhibir el crecimiento al inhibir la traducción o la replicación. C) Tipo III: la antitoxina es un RNAm no codificante y se une con la toxina endoribonucleasa (RNasa), lo que resulta en la formación de complejos de RNA-toxina, que inhiben la actividad de la toxina. D) Tipo IV: la proteína antitoxina

estabiliza los filamentos bacterianos, mientras que la proteína toxina los desestabiliza; en ausencia de la antitoxina, esta desestabilización mediada por toxinas inhibe la división celular. E) Tipo V: la antitoxina GhoS es una RNasa específica para el RNAm de la toxina; En condiciones de estrés, el RNAm de la antitoxina es degradado por la toxina MqsR, lo que resulta en la traducción de GhoT y lisis de membrana. F) Tipo VI: la antitoxina SocA es una proteína adaptadora que se une a la toxina SocB para promover su degradación por ClpXP. Cuando no se degrada, la toxina inhibe la replicación del DNA (Modificado de Page and Peti, 2016).

La forma de regulación de los sistemas TA tipo II por parte de la antitoxina puede ser de dos formas: la primera cuando se inhibe la actividad de la toxina mediante la formación de un complejo toxina-antitoxina, la segunda cuando el complejo toxina-antitoxina o la antitoxina se unen a la región promotora del operón inhibiendo la transcripción de ambos componentes, esto dependerá de las proporciones equimolares entre la antitoxina y la toxina. (Jensen and Gerdes, 1995). El sistema TA tipo II es el único tipo en el que la proteína antitoxina se une directamente a la toxina, formando un complejo proteína-proteína, que secuestra la toxina y neutraliza su actividad (Goeders and Van Melderren, 2014).

Originalmente los sistemas TA tipo II se agruparon en familias basadas en la similitud de secuencia y estructura de los genes, y se supone que cada familia de toxinas se asocia con una familia antitoxina específica. Sin embargo, ahora se ha hecho evidente que existen muchos sistemas híbridos donde un locus TA contiene una toxina de una clase y una antitoxina de otra clase. Recientemente, se sugirió clasificar las toxinas en diez familias: *ccdAB*, *mazEF*, *vapBC*, *phd/doc*, *parDE*, ϵ - ζ , *higBA*, *relBE*, *hipBA* y *hicAB*, las cuales pueden ser codificadas por plásmidos o cromosomas (Gerdes *et al*, 2005; Fivian-Hughes and Davis, 2010; Leplae *et al.*, 2011). Por otro lado las antitoxinas se clasificaron en 20 familias, por ejemplo las antitoxinas HTH, las cuales tienen un motivo Helice-Giro-Helice (HTH; helix-turn-helix) de unión de DNA y neutraliza a su toxina afín a nivel transcripcional; además, se identificaron cuatro familias de toxinas 'solitarias' que no están flanqueadas por antitoxinas y que mostraron inhibición del crecimiento si se sobreexpresan en *E. coli* (Leplae *et al*, 2011). El sistema TA tipo II *relEB* es uno de los más estudiados, se ha

reportado que la toxina RelE es capaz de cortar el RNAm en el sitio A ribosómico, por lo cual inhibe la traducción de proteínas, y su antitoxina RelB, se une e inhibe RelE, y está regula la transcripción a través de la unión del operador y la cooperatividad condicional controlada por RelE (Bøggild *et al*, 2012).

❖ TIPO III

Recientemente, un sistema TA tipo III se descubrió en un plásmido de *Erwinia carotovora* (Fineran *et al.*, 2009). Al igual que en los sistemas tipo I y II, la toxina ToxN es una proteína mientras que la antitoxina ToxI es una molécula de RNA. En su modo de acción, ToxI no inhibe la expresión de la toxina, sino más bien inhibe la actividad de la toxina, mediante el secuestro de la toxina por la formación de un complejo (Pecota and Wood, 1996; Blower *et al.*, 2011). Curiosamente, el sistema de tipo III se descubrió como un sistema de protección de bacterias contra infecciones de bacteriófagos. Se detectó un pequeño número de homólogos de toxinas tipo III tanto en bacterias Gram-positivas y Gram-negativas (Fineran *et al.*, 2009; Hayes and Van Melderren, 2011).

❖ TIPO IV

Se propuso una designación de sistema TA tipo IV para el módulo TA yeeU/yeeY (también llamado ctbA/ctbB) de *E. coli*. El análisis funcional de este sistema TA reveló que la toxina YeeV interactúa con MreB (actina) y FtsZ (tubulina) y por lo tanto interfiere con su polimerización (Masuda *et al.*, 2012). Mientras que la toxina y la antitoxina de todas los demás sistemas TA interactúan a nivel del RNA o de la

proteína, la toxina y la antitoxina de este tipo de sistema TA no interactúan directamente (Unterholzner *et al.*, 2013).

❖ TIPO V

Recientemente, el sistema TA GhoS/GhoT de *E. coli* se designó como un sistema tipo V. La proteína antitoxina GhoS tiene una actividad de endoribonucleasa específica de la secuencia para la escisión del RNAm de la toxina GhoT, por lo tanto evita la traducción de la toxina (Wang *et al.*, 2012). Este es el único caso descrito en el que la antitoxina tiene una actividad enzimática (Lobato-Márquez and Díaz-Orejas, 2016).

❖ TIPO VI

SocAB es el único sistema TA tipo VI encontrado en la actualidad (Aakre *et al.*, 2013). La antitoxina SocA es una proteína adaptadora que se une a la toxina SocB. A diferencia de los módulos TA tipo II, SocA no neutraliza a SocB directamente, sino que promueve su degradación por una proteasa específica (Lobato-Márquez and Díaz-Orejas, 2016).

3. Mecanismos de acción de los sistemas TA

En los sistemas TA, las moléculas de toxina (proteínas) son considerados como moléculas dañinas para las células (Kunin and Ouzounis, 2003). Los tiempos de vida de las moléculas de toxina y antitoxina son muy diferentes en las células: las moléculas de toxina tienden a ser estables mientras que las moléculas de antitoxina tienden a ser inestables. Por lo tanto, para evitar los efectos tóxicos de las moléculas de toxina, las moléculas de antitoxina son producidas continuamente (Aizenman *et*

al., 1996). Las dianas celulares de estas toxinas son muy diversas e incluyen la replicación del DNA, la estabilidad del RNAm, la síntesis de proteínas, la biosíntesis de la pared celular, y la síntesis de ATP. Los sistemas TA pueden jugar un papel en la supervivencia celular bajo condiciones de estrés tales como falta de aminoácidos, el tratamiento con antibióticos, cambio de temperatura y el estrés oxidativo, a pesar de que los sistemas TA no son esenciales para el crecimiento celular normal (Van Melderren and Saavedra De Bast, 2009; Yamaguchi *et al.*, 2011).

4. Sistemas TA cromosómicos

Los genes TA también se encuentran localizados en los cromosomas bacterianos, aunque su papel exacto en este entorno se debate (Keren *et al.*, 2004; Buts *et al.*, 2005; Gerdes *et al.*, 2005; Engelberg-Kulka *et al.*, 2006; Szekeres *et al.*, 2007; Nariya and Inouye, 2008). Un análisis completo de los genomas de procariontes han puesto de relieve la diversidad en la distribución de los sistemas TA (Anantharaman and Aravind, 2003; Pandey and Gerdes, 2005; Sevin and Barloy-Hubler, 2007). Sin embargo, se ha descrito que no existe correlación entre el número de sistemas TA, el estilo de vida, la pertenencia a un phylum, o la tasa de crecimiento.

Algunos genomas tales como los de *Nitrosomonas europaea*, *Sinorhizobium meliloti*, y *Mycobacterium bovis* contienen más de 50 sistemas TA putativos (Pandey and Gerdes, 2005). Algunos otros contienen ninguno o muy pocos (menos de tres) sistemas TA putativos, como *Rickettsia prowazeki*, *Campylobacter jejuni*, o *Bacillus subtilis*. La presencia de al menos 33 sistemas TA en *E. coli* y más de 60 sistemas TA en *Mycobacterium tuberculosis* sugiere que los sistemas TA están implicados no sólo en la fisiología bacteriana normal, sino también en la patogenicidad de las

bacterias (Leplae *et al.*, 2011). Mediante un análisis bioinformático se encontró que en el genoma de *Salmonella typhimurium* están presentes 11 sistemas TA de tipo II, de los cuales varios se conservan en otras cepas patógenas de la misma especie pero están ausentes en cepas no patógenas, lo que sugirió que ciertos sistemas TA podrían tener un papel en la patogenicidad de *Salmonella*. Adicionalmente, se demostró que la actividad del sistema *sehAB* de *S. typhimurium*, juega un papel en la virulencia en ratones (De la Cruz *et al.*, 2013).

5. *Pseudomonas aeruginosa*

P. aeruginosa es una bacteria Gram-negativa perteneciente a la rama de las proteobacterias, misma a la que pertenecen las enterobacterias (Pace, 1997). Se encuentra ampliamente distribuida en la naturaleza, se puede aislar de muestras de suelo, pantanos y hábitats marinos costeros, así como de plantas y animales. Ésta puede utilizar el nitrógeno como un aceptor de electrones para respirar bajo condiciones anaeróbicas, tiene un mínimo de requerimiento nutrimental y puede crecer a temperaturas arriba de 42°C (Pier, 2005), además, es capaz de prosperar en múltiples nichos. Se ha reportado el aislamiento de *P. aeruginosa* de ambientes tan inhóspitos como son el combustible de aviones, soluciones de clorhexidina y el jabón (Hardalo and Edberg, 1997).

P. aeruginosa puede infectar a diferentes organismos, incluyendo levaduras (Hogan and Kolter, 2002), al nematodo *Caenorhabditis elegans* (Mahajan-Miklos *et al.*, 1999), insectos (Jander *et al.*, 2000), plantas (Elrod and Braun, 1942; Rahme *et al.*, 1995) y mamíferos (Glazebrook *et al.*, 1978; Hammer *et al.*, 2003). Además, es capaz de resistir a varios antibióticos y desinfectantes. Así mismo, se ha descrito

que esta bacteria emplea los mismos determinantes de virulencia para infectar diferentes hospederos, desde plantas hasta humanos (Fajardo *et al.*, 2008). Es el patógeno más comúnmente asociado con la mortalidad de pacientes con fibrosis quística (Battle *et al.*, 2008).

Pseudomonas aeruginosa es uno de los patógenos oportunistas más importantes en humanos. Pertenece al grupo ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* y *Enterobacter spp.*), que es un grupo de bacterias resistentes a los antibióticos que son causas comunes de infecciones nosocomiales potencialmente mortales (Pendleton *et al.*, 2013). Los pacientes con enfermedades crónicas o sistemas inmunes comprometidos debido a inmunodeficiencia o quemaduras tienen un mayor riesgo de infección por *P. aeruginosa* (Quick *et al.*, 2014). El éxito de las infecciones por *P. aeruginosa* se debe en gran medida a la producción de factores de virulencia para provocar enfermedades tanto en plantas como en animales y su capacidad para formar biopelículas (Rahme *et al.*, 2000). La producción de varios factores de virulencia en *P. aeruginosa* se controla según la densidad celular a través de dos sistemas de detección de quórum sensing (QS). La red QS de *P. aeruginosa* está organizada en una jerarquía de varias capas que consta de al menos cuatro mecanismos de señalización interconectados, que incluyen las, iqs, pqs y rhl (Lee and Zhang, 2015).

En el 2000, se obtuvo la secuencia completa del genoma de la cepa PAO1 de *P. aeruginosa*, que fue aislada de un paciente con otitis media. Esta secuencia ya se encuentra disponible en internet (www.pseudomonas.com) y su análisis fue

publicado (Stover *et al.*, 2000). El tamaño aproximado del único cromosoma circular (www.pseudomonas.com) que forma el genoma de esta bacteria es de cerca de 6.3 millones de pares de bases y un total de 5570 marcos de lectura abiertos. PAO1 es una cepa estándar del laboratorio y es ampliamente usada en estudios de investigación básica (Holloway, 1955). Cuando fue secuenciado el genoma de ésta cepa se reveló que este posee un gran número de genes que están involucrados en la regulación, catabolismo, transporte y expulsión de factores de virulencia, además de codificar proteínas de membrana involucradas en adhesión, motilidad y eflujo de antibióticos, al igual que varios sistemas putativos de quimiotaxis (Stover *et al.*, 2000).

5.1 Factores de virulencia

Los patógenos bacterianos infectan una amplia variedad de hospederos, en todos los casos, los patógenos pueden tener la habilidad para reconocer, asociarse con la explotación de la reserva de nutrientes y combatir las respuestas de defensas de su hospedero específico (Rahme *et al.*, 2000), para acoplarse a estas tareas, los patógenos usan un arsenal extenso de factores relacionados a virulencia. Las bacterias a lo largo de la evolución han adquirido características que les permiten invadir el ambiente del hospedero, expresar receptores superficiales especializados para su adhesión (Meylan *et al.*, 2006), permanecer en estos sitios a través de procesos de colonización, evadir al sistema inmune (Diacovich and Gorvel, 2010) y finalmente causar daño tisular con el fin de lograr acceso a fuentes de nutrientes necesarios para su crecimiento y reproducción. Por lo que el factor o determinante

de virulencia es un componente microbiano que favorece el crecimiento o sobrevivencia durante la infección (Tortora *et al.*, 2007; Wu *et al.*, 2008).

Usando a la cepa de *Pseudomonas aeruginosa* PA14 se evidenció que ésta bacteria usa un subconjunto de factores de virulencia para provocar la enfermedad tanto en plantas (*Arabidopsis thaliana*), como en animales (Ratones) (Rahme *et al.*, 1995; He *et al.*, 2004), en el nematodo *Caenorhabditis elegans* (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999; Tan and Ausubel, 2000) y en el insecto *Galleria melonella* (Jander *et al.*, 2000), así como en *Drosophila melanogaster* (Lutter *et al.*, 2012). Los factores de virulencia de *P. aeruginosa* facilitan la invasión de tejidos y la propagación, en la que se incluyen al pilus, al flagelo, endotoxinas, exotoxinas, factores de permeabilidad vascular, y una variedad de enzimas secretadas (Ben *et al.*, 2011). Las proteasas de *P. aeruginosa* degradan una gran variedad de proteínas del hospedero y tienen un efecto destructivo directo en los tejidos (Kawaharajo *et al.*, 1975; Holder and Neely, 1991), un ejemplo es la elastasa (LasB), una proteína metaloproteasa con baja especificidad de sustrato, que degrada las proteínas del hospedero, tales como elastina, colágeno, transferinas e inmunoglobulinas (Galloway, 1991). La elastasa actúa de acuerdo con las proteasas LasA y las proteasas alcalinas que causan una elastolisis eficiente, la cual es requerida para el daño del tejido asociado con la patogénesis de *P. aeruginosa* (Peters and Galloway, 1990). Otro ejemplo de factor de virulencia es la cápsula de alginato de *P. aeruginosa*, la cual juega un rol esencial en la infección pulmonar crónica en pacientes con fibrosis quística (Ohman *et al.*, 1980; Zielinski *et al.*, 1991).

Algunos de los factores de virulencia son regulados por factores externos o internos de la célula, por ejemplo, la fosfolipasa C es regulada por la disponibilidad de fosfato (Pritchard and Vasil, 1986; Shortridge *et al.*, 1992), mientras que la exotoxina A y la elastasa son reguladas por la concentración de hierro en el medio de crecimiento (Grant and vasil, 1986). Además, la producción de un gran número de exoproductos, incluyendo elastasa, proteasa alcalina, la proteasa LasA, hemolisina, piocianina y ramnolipidos son dependientes de la densidad celular y son regulados por la llamada cascada de quorum-sensing (Pesci and Iglewski, 1997), esta cascada es mediada por homoserina lactonas de baja masa molecular que son sintetizadas por los productos de los genes *lasI* y *rhII*, así regulando la producción de una variedad de factores específicos relacionados a virulencia, involucrando al quorum-sensing en la diferenciación de biofilms en *P. aeruginosa* (McLean *et al.*, 1997; Davies *et al.*, 1998). Otro factor de virulencia regulado por quorum sensing es la piocianina, una fenazina color azul verdoso (Fuqua *et al.*, 1994; Pesci and Iglewski, 1997), este metabolito secundario tiene actividad microbiana en contra de varias especies de bacterias, hongos y protozoarios, teniendo como cualidad atribuida la de potencial activo redox (Hassan and Fridovich, 1980; Sorensen *et al.*, 1983).

5.2 Sistema Quorum Sensing

La comunicación célula a célula por medio de moléculas difusibles permite que las bacterias desencadenen respuestas coordinadas para lograr resultados que de otro modo resultarían imposibles para una sola célula (Jiménez *et al.*, 2012). El Quorum-sensing (QS) es considerado un mecanismo de comunicación entre bacterias que permite regular procesos importantes como la formación de biopelículas, la

producción de metabolitos secundarios, la expresión de mecanismos de resistencia al estrés y la expresión de factores de virulencia (Antunes *et al.*, 2010). Tales fenómenos se llevan a cabo a través de mecanismos de autoinducción de señales químicas presentes tanto en bacterias Gram negativas como en Gram positivas (Whitehead *et al.*, 2001). Las N-Acyl homoserina lactonas (AHLs) fueron las primeras señales bacterianas célula a célula que fueron descubiertas, además de que son las moléculas de comunicación más estudiadas (Jiménez *et al.*, 2012). Un gran número de bacterias Gram negativas producen y usan éstas señales para controlar y regular la expresión de genes de una manera dependiente de la densidad celular (Fuqua and Greenberg, 1998; Whitehead *et al.*, 2001; Withers *et al.*, 2001). En general, las AHLs consisten en ácidos grasos de longitud variable, ligados vía un enlace peptídico a la homoserina lactona. En *Pseudomonas aeruginosa* coexisten dos sistemas AHL: el sistema Las y Rhl. El sistema Las produce y responde a N-3-oxo-dodecanoil homoserina lactona (3-oxo-C₁₂-HSL), la cual es producida por la sintasa LasI y reconocida por el regulador transcripcional LasR (Passador *et al.*, 1993; Rampioni *et al.*, 2007). El sistema Las, controla la producción de múltiples factores de virulencia involucrados en infecciones agudas y daño a las células hospederas, incluyendo a LasA, LasB, elastasas, exotocina A y la proteasa alcalina (Toder *et al.*, 1991; Gambello *et al.*, 1993; Passador *et al.*, 1993). El segundo sistema, el Rhl, produce y responde a N-butanoil homoserina lactona (C₄-HSL) (Pearson *et al.*, 1995), ésta molécula es generada por la sintasa RhlI y es reconocida por el regulador transcripcional RhlR, este complejo regula la expresión de varios genes, incluyendo genes responsables de la producción de rhamnolipidos, y reprimiendo genes de respuesta para ensamblaje y función del sistema de

secreción tipo III, el mayor determinante de infecciones en humanos, el cual permite liberar proteínas tóxicas dentro del citoplasma de células eucariotas (Bleves *et al.*, 2005). Existe una relación entre los sistemas Las y Rhl: el sistema Las, controla al sistema Rhl, el complejo 3-oxo-C₁₂-HSL-LasR directamente sobre regula la transcripción de *rhlR* (Latifi *et al.*, 1996) (**Figura 3**).

Se ha reportado que existe una correlación significativa entre los sistemas QS y los sistemas TA en aislados clínicos de *P. aeruginosa* con una alta capacidad de formación de biopelículas (Hemati *et al.*, 2014).

5.3 Sistemas TA de *P. aeruginosa*

Williams *et al.*, (2011) encontraron la presencia de los sistemas TA RelEB, HigAB y ParDE en 42 aislados clínicos de *P. aeruginosa*, demostraron la transcripción de los genes de los sistemas encontrados, concluyendo que estos tres sistemas TA son altamente prevalentes y se transcriben en cepas de *P. aeruginosa*, sugiriendo que esta bacteria es susceptible a las estrategias antibacterianas basadas en la activación de los sistemas TA para que la toxina liberada mate a la célula bacteriana que contiene a dicho sistema. Además, en un trabajo se mostró que 174 aislados clínicos de *P. aeruginosa*, los sistemas TA relBE y higBA eran universales, pero el sistema parDE era menos frecuente (Savari *et al.*, 2016).

En *P. aeruginosa* PAO1 se han reportado genes que codifican a probables sistemas TA tipo II RelBE, ParDE y HigBA, sin embargo estos no se han caracterizados (Pandey and Gerdes, 2005). Un sistema TA prominente que se encuentra en muchos patógenos es HigB/HigA, en el que la antitoxina HigA enmascara la toxicidad de la toxina HigB.

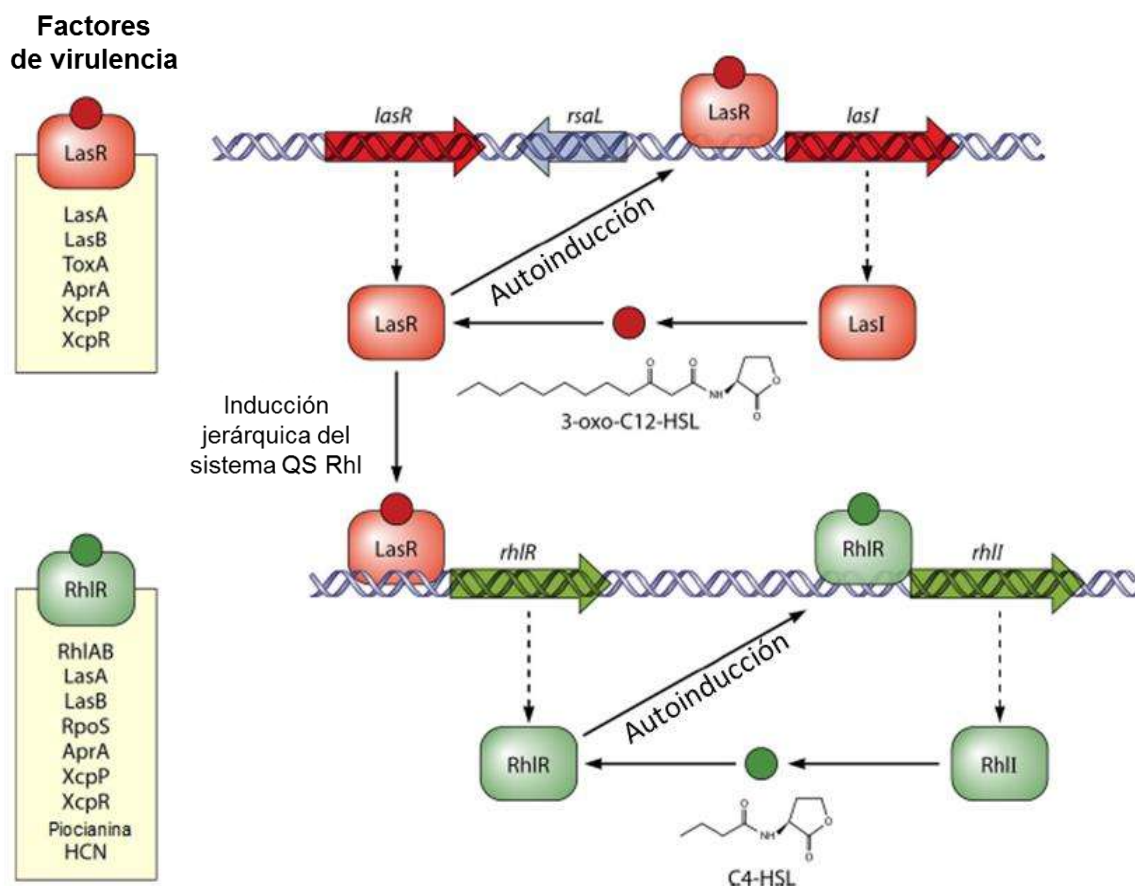


Figura 3. Regulación de la virulencia y las interacciones entre los sistemas Las y Rhl de Quorum sensing de *Pseudomonas aeruginosa*. Después de que se produjo una concentración umbral del 3-oxo-C12-HSL, el complejo 3-oxo-C12-HSL-LasR se une a las regiones promotoras de múltiples genes, activando o reprimiendo la transcripción. Entre los genes regulados positivamente por este complejo son *lasI*, que aumenta la producción de 3-oxo-C12-HSL (efecto de autoinducción), y *rhIR*, lo que aumenta la producción de *rhl*, regulador de respuesta de RhIR, activando una segunda vía de AHL en una etapa anterior. Los factores de virulencia regulados por cada respectivo complejo receptor-ligando se detallan en los recuadros de la izquierda (Jiménez *et al.*, 2012).

Los genes para el sistema TA HigB/HigA se encuentran en el plásmido Rts1 originalmente aislado de *Proteus vulgaris* y se encuentra en los cromosomas de los patógenos *Vibrio cholerae* (Gerdes *et al.*, 2005), *Acinetobacter baumannii* (Jurenaite *et al.*, 2013), *S. typhimurium* (De la Cruz *et al.*, 2013), *Yersinia pestis* (Goulard *et al.*, 2010), *M. tuberculosis* (Schuessler *et al.*, 2013), *E. coli* CFT073 (Pandey and Gerdes, 2005). Además HigBA está muy difundido en aislamientos clínicos de *P. aeruginosa* (Williams *et al.*, 2011).

P. aeruginosa PA14 (es una bacteria altamente virulenta) posee un sistema TA tipo II en el que la antitoxina HigA enmascara la actividad de RNasa de la toxina HigB a través de la unión directa. Además la toxina HigB reduce la producción de los factores de virulencia como piocelina, piocianina y la formación de biopelícula (Wood and Wood, 2016).

IV. ANTECEDENTES

1. Plásmido pUM505

El plásmido conjugativo pUM505 fue aislado de una cepa clínica de *P. aeruginosa* de un paciente hospitalizado (Cervantes and Ohtake, 1988). En el 2011, se realizó la secuenciación y el análisis de la secuencia del plásmido; y se encontró que es un replicón circular de 123,332 pares de bases (pb) donde se identificaron 138 regiones codificantes (**Figura 4**) (Ramírez-Díaz *et al.*, 2011).

El plásmido pUM505 posee dos islas genómicas, la primera de ~67 kb corresponde a una isla de patogenicidad (PAI) y la segunda, de ~31 kb, corresponde a una isla genómica que contiene genes de resistencia a metales. Las PAIs son típicamente grandes regiones genómicas (10-200 kb) que están presentes en los genomas de las cepas patógenas en donde se localizan genes que codifican a factores de virulencia de bacterias patógenas, como adhesinas, toxinas, y los sistemas de secreción de proteínas (Hacker and Kaper, 2000). La PAI de pUM505 consiste en 78 marcos de lectura abiertos (*orfs* 1-51 y 112-138), posee genes implicados en la replicación del plásmido, la transferencia por conjugación y genes relacionados con virulencia (Ramírez-Díaz *et al.*, 2011).

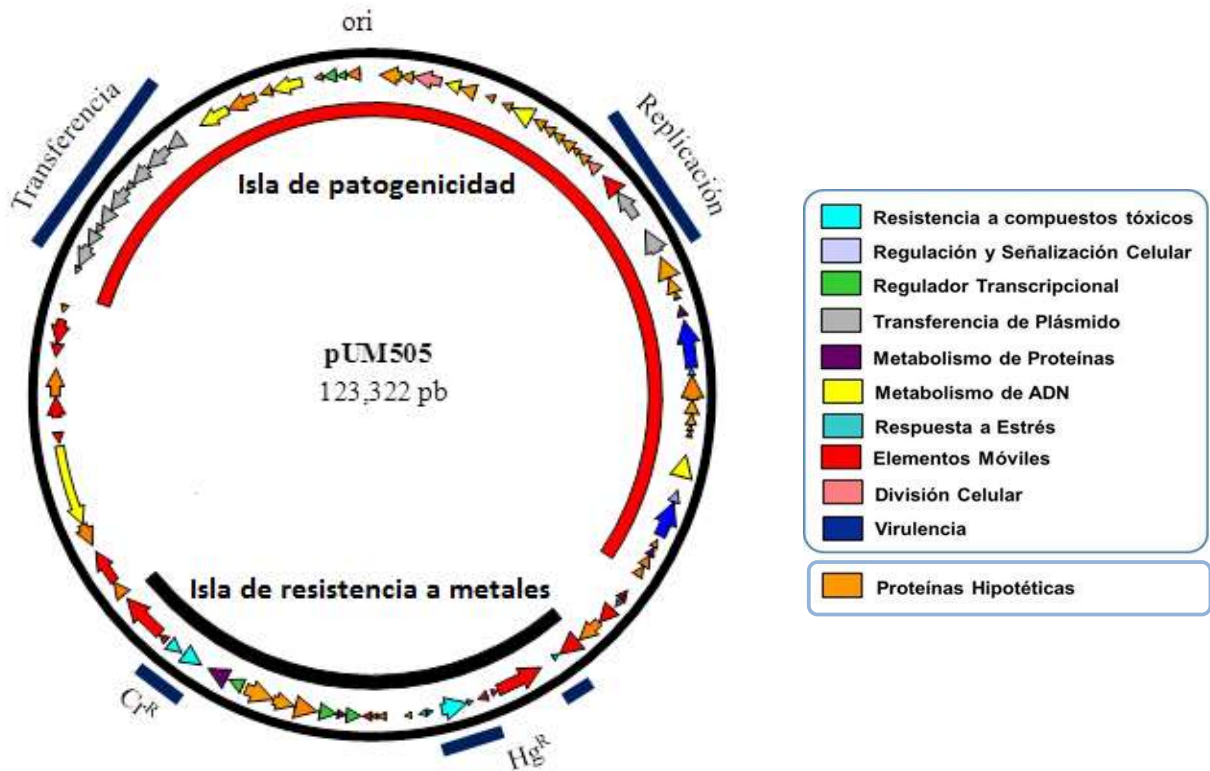


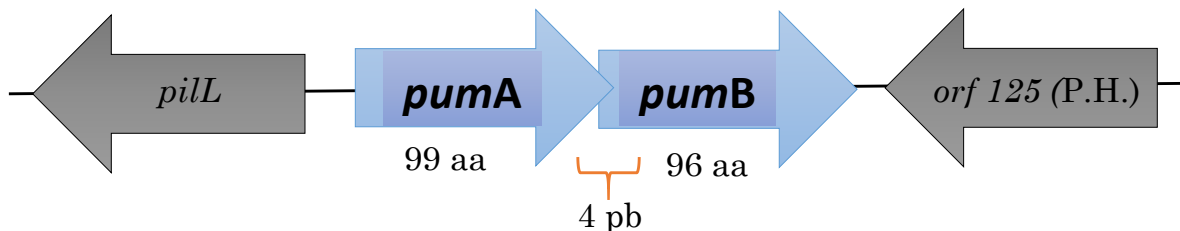
Figura 4. Mapa genético del plásmido pUM505 de *P. aeruginosa*. Las regiones codificantes se muestran con flechas o puntas de flecha, indicando la dirección de la transcripción. Las posibles funciones asignadas de las proteínas codificantes se agrupan de acuerdo a los diferentes colores mostrados en el cuadro de la derecha, indicando también las proteínas hipotéticas. La isla de patogenicidad (PAI) se muestra con una barra roja y la isla de resistencia a metales pesados con una barra negra. Se indica el origen de replicación (ori) y con barras azules las regiones relacionadas con la transferencia, replicación, resistencia a mercurio (Hg^R) y resistencia a cromato (Cr^R) (Modificado de Ramírez-Díaz *et al.*, 2011).

Se ha sugerido que pUM505 podría proporcionar a las cepas de *Pseudomonas* una amplia variedad de rasgos adaptativos que pueden comprender factores selectivos para la distribución y prevalencia de este plásmido en diversos entornos (Ramírez-Díaz *et al.*, 2011).

1.1 Sistema TA PumAB

La PAI del plásmido pUM505 posee los *orfs* 123 y 124 (renombrados *pumA* y *pumB*, respectivamente) y codifican para un sistema toxina-antitoxina. El gen *pumA* codifica una proteína 55% idéntica a la proteína RelE (componente tóxico) del sistema TA de *Klebsiella pneumoniae*. El gen *pumB* codifica un probable regulador transcripcional 82% similar a la proteína de la antitoxina HTH (Helix-Turn-Helix) de *Pseudomonas alcaligenes*, por lo que fue clasificado como un sistema TA tipo II (**Figura 5**). El sistema PumAB mostró ser un sistema TA no convencional, dado que los genes del operón *pumAB* muestran una organización génica diferente a la mayoría de los sistemas TA tipo II, en donde usualmente el gen que codifica a la antitoxina precede al gen de la toxina (Hernández-Ramírez, 2015). Además, las proteínas del sistema PumAB no son canónicas, PumA es una toxina tipo RelE, toxina que forma parte de sistemas TA en conjunto con la antitoxina RelB; mientras que la proteína PumB es similar a las antitoxinas tipo HTH. Los genes *pumA* y *pumB* son expresados a través de un RNAm bicistrónico, indicando que ambos genes se co-expresan, lo que sugirió que forman un operón (Hernández-Ramírez, 2015). El sistema PumAB es capaz de conferir la estabilidad de plásmidos en *E. coli*, de manera similar a otros sistemas TA codificados en plásmidos.

A)



B)

Proteína	Identidad (%)	Proteína	Tipo de Sistema TA	Organismo
PumA	55	RelE (Toxina)	II	<i>Klebsiella pneumoniae</i>
PumB	82	HTH (Antitoxina)	II	<i>Pseudomonas alcaligenes</i>

Figura 5. Sistema TA PumAB en el plásmido pUM505 de *P. aeruginosa*. A)

Arreglo genómico del probable sistema TA PumAB en el plásmido pUM505. Con flechas azules se indican los genes *pumA* (RelE) y *pumB* (HTH) con una región sobrepuesta de 4 pb. Se indica el tamaño predicho en aa de las proteínas PumA (toxina) y PumB (antitoxina) codificadas, y en gris se muestran los genes aledaños.

P.H.: Proteína Hipotética. B) Características de los productos proteicos predichos después de hacer el cluster (Hernández-Ramírez, 2015).

La transferencia y sobreexpresión de *pumA* en *E. coli* BL21 generó la inhibición del crecimiento bacteriano, sugiriendo que PumA podría generar un efecto tóxico en la bacteria (Hernández-Ramírez, 2015). La inhibición del crecimiento bacteriano ha sido descrita como una función de las toxinas. En *E. coli* K-12 (MG1655), las toxinas RelE, MazF, YeeV e YkfI inhibieron el crecimiento bacteriano cuando se sobreexpresaron ectópicamente (Ahidjo *et al.*, 2011; Pedersen *et al.*, 2002).

Los sistemas TA encontrados en cromosoma han sido relacionados con el aumento de la virulencia de sus hospederos bacterianos, incluyendo *M. tuberculosis* (Sala *et al.*, 2014) y *E. coli* (Norton and Mulvey, 2012). Interesantemente, la transferencia del gen *pumA* aumentó la virulencia de *P. aeruginosa*, ya que transformantes con el gen generaron la muerte de ratones además de aumentar la invasión de *P. aeruginosa* en tejidos como corazón, pulmón y riñón (Hernández-Ramírez, 2015) (**Figura 6**). Estos datos sugirieron que la proteína PumA podría actuar como un factor de virulencia para la bacteria. La transferencia conjunta de los genes *pumA* y *pumB* a *P. aeruginosa* no generó el aumento de la virulencia de las transformantes, sugiriendo que PumB neutraliza los efectos tóxicos de PumA (Hernández-Ramírez, 2015). Varios sistemas TA cromosómicos causan que sus hospederos bacterianos generen una mayor virulencia contra los animales, incluidos los sistemas de TA de *E. coli* (Norton and Mulvey, 2012), *Salmonella typhimurium* (De la Cruz *et al.*, 2013), *M. tuberculosis* (Sala *et al.*, 2014), *L. interrogans* (Komi *et al.*, 2015); sin embargo, esta relación no había sido descrita para elementos de sistemas TA codificados por plásmidos.

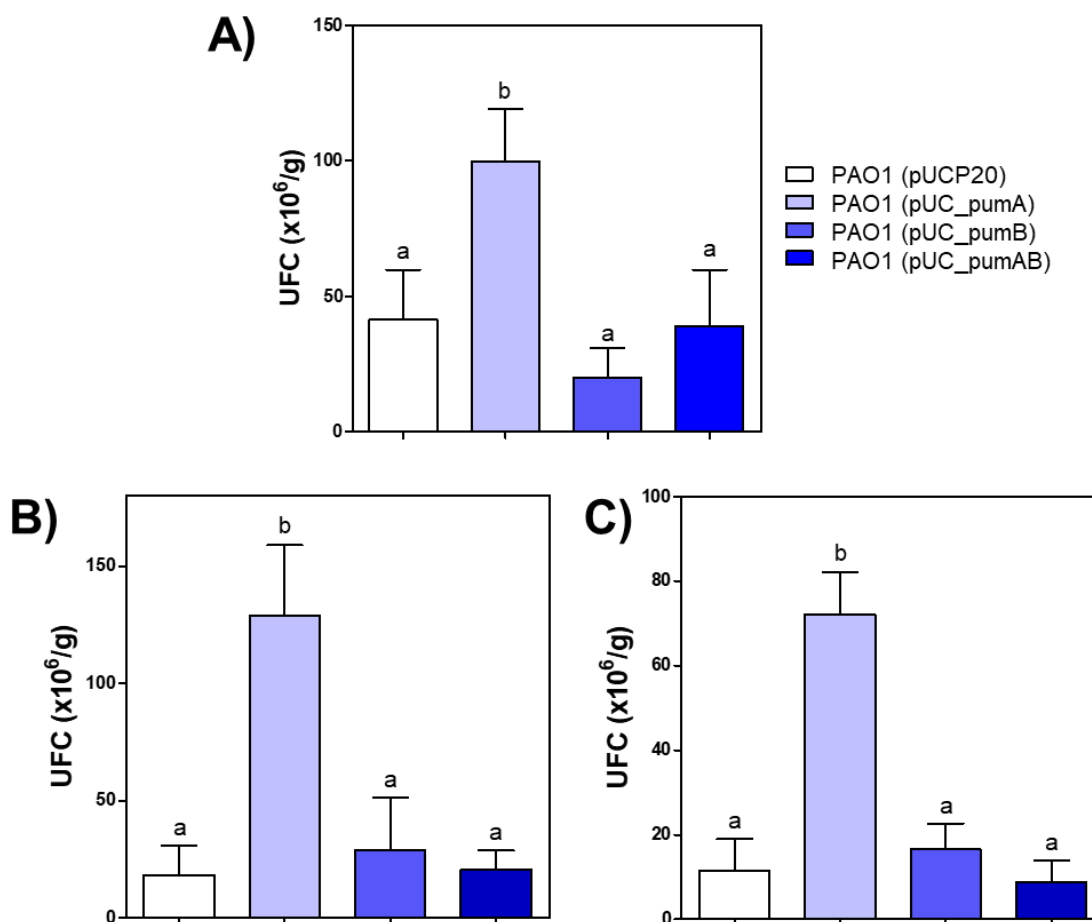


Figura 6. Invasividad de *P. aeruginosa* en ausencia o presencia de los genes *pumA* y *pumB* en órganos de ratón. Se inocularon ratones por vía intravenosa con la diferentes cepas, después de 48 h se extrajeron diferentes tejidos y se determinó las UFC obtenidas de placas de Agar Nutritivo a 37°C. A) UFC en corazón; B) UFC en pulmones y C) UFC en riñones. Se grafican barras en diferentes colores dependiendo de la cepa utilizada. Se muestran las barras de error estándar de la media, n= 7. $P < 0.05$, pos hoc, prueba de Tukey. (Modificado de Hernández-Ramírez, 2015).

V. JUSTIFICACIÓN

Los sistemas TA se encuentran ampliamente distribuidos en plásmidos y cromosomas bacterianos, principalmente en bacterias patógenas. Los genes *pumA* y *pumB* del plásmido pUM505 codifican un sistema TA tipo II que participa en la estabilidad plasmídica. La proteína PumA del sistema PumAB incrementa la virulencia de *P. aeruginosa*, una de las principales bacterias causantes de infecciones oportunistas en humanos que produce factores de virulencia regulados por el sistema de Quorum sensing. Además, la virulencia conferida por PumA es neutralizada por función de la antitoxina PumB. Los sistemas TA tipo II pueden ser regulados de dos formas, a nivel transcripcional con la unión de la antitoxina a la región promotora-operadora y/o con la inhibición de la actividad de la toxina formando un complejo Toxina-Antitoxina. Por lo que es de interés caracterizar la forma de regulación del sistema TA de pUM505.

VI. HIPÓTESIS

El sistema TA PumAB de pUM505 que confiere virulencia mediante la proteína PumA (toxina), de forma dependiente de quorum sensing, es regulado por la proteína PumB (Antitoxina) a nivel transcripcional y mediante la interacción con PumA (toxina).

VII. OBJETIVOS

General

Caracterizar el mecanismo de regulación del sistema TA del plásmido pUM505 por parte de la antitoxina PumB.

Específicos

1. Determinar si la antitoxina PumB neutraliza a la toxina PumA mediante la inhibición de su actividad.
2. Determinar si la antitoxina PumB es un regulador transcripcional del operón *pumAB*.
3. Establecer si el sistema TA PumAB es regulado por el sistema de Quorum sensing.

VIII. RESULTADOS

Los resultados generados durante la realización del presente proyecto se presentan en tres capítulos:

CAPITULO I. Artículo: Plasmid pUM505 encodes a Toxin-Antitoxin system conferring plasmid stability and increased *Pseudomonas aeruginosa* virulence.

CAPITULO II. Artículo: Role of PumB Antitoxin as a Transcriptional Regulator of the PumAB Type-II Toxin-Antitoxin System and its Endoribonuclease Activity on PumA (Toxin) transcript.

CAPITULO III. Artículo: Virulence conferred by PumA toxin from the plasmid encoded PumAB Toxin-Antitoxin System is Regulated by Quorum System.

CAPITULO I

Artículo: Plasmid pUM505 encodes a Toxin-Antitoxin system conferring plasmid stability and increased *Pseudomonas aeruginosa* virulence.



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Plasmid pUM505 encodes a Toxin–Antitoxin system conferring plasmid stability and increased *Pseudomonas aeruginosa* virulence



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ABSTRACT

Pseudomonas aeruginosa plasmid pUM505 possesses a pathogenicity island that contains the *pumAB* genes that encode products with sequence similarity to Toxin–Antitoxin (TA) modules. RT-PCR assays on the overlapping regions of the *pumAB* genes generated a bicistronic messenger RNA, suggesting that they form an operon. When the *pumAB* genes were cloned into the pJET vector, recombinant plasmid pJET-*pumAB* was maintained under nonselective conditions in *Escherichia coli* cells after six daily subcultures, whereas pJET without *pumAB* genes was lost. These data indicate that *pumAB* genes confer post-segregational plasmid stability. In addition, overexpression of the PumA protein in the *E. coli* BL21 strain resulted in a significant growth inhibition, while BL21 co-expressing the PumA and PumB proteins did not show growth inhibition. These results indicate that *pumAB* genes encode a TA system where the PumB protein counters the toxic effects of the PumA toxin. Furthermore, *P. aeruginosa* PAO1 transformants with the *pumA* gene increased *Caenorhabditis elegans* and mouse mortality rate and improved mouse organ invasion, effects neutralized by the PumB protein. Moreover, purified recombinant His-PumA protein decreased the viability of *C. elegans*, indicating that the PumA protein could act as a toxin. These results indicate that PumA has the potential to promote the PAO1 virulence against *C. elegans* and mice when is expressed in absence of PumB. This is the first description, to our knowledge, of a plasmid-encoded TA system that confers plasmid stability and encoded a toxin with the possible ability to increase the *P. aeruginosa* virulence.

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1. Introduction

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that is ubiquitously distributed in different terrestrial, aquatic, animal, human, and plant environments [38]. *P. aeruginosa* is one of the most important opportunistic pathogens in humans. Patients with chronic diseases or with compromised immune systems, such as immunodeficiency or burns, are at greater risk of infection [32]. In addition, *P. aeruginosa* uses a shared subset of virulence factors to elicit disease in both plants and animals [33]. The conjugative

plasmid pUM505 is an IncI type of 123 kilobases (kb) in size, originally isolated from a *P. aeruginosa* clinical strain, which confers resistance to chromate ions and inorganic mercury [10]. Sequence analysis revealed that pUM505 carries several adaptive genes, including *umuD*, which functions as a transcriptional regulator of SOS genes [14], as well as several genes related to virulence [35]. It has been suggested that pUM505 could provide *Pseudomonas* strains with a wide variety of adaptive traits that can comprise selective factors for the distribution and prevalence of this plasmid in diverse environments [34]. The predicted products of Open Reading Frames (ORF) 123 and 124 from pUM505 exhibit sequence similarity to Toxin–Antitoxin (TA) systems [34]. TA systems are small genetic modules found in bacterial mobile genetic elements, as well as in bacterial chromosomes, which currently are grouped into the six types of TA systems [23,28]. The toxins of all characterized

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bacterial TA systems are proteins, while the antitoxins are either proteins or small RNA (sRNA) [41]. Antitoxins are more labile and more readily degraded under stress conditions than toxins, allowing toxins to exert their toxic effects [44]. In TA systems, toxins are regarded as harmful molecules to cells due to their cell-growth inhibitory effects, thereby conferring growth stasis in stressful environments. The antitoxin molecules are usually binding partners of toxins, either on a gene or on a protein level, and they regulate the activity of the toxin [29]. Although TA systems were originally discovered in plasmids, bacterial and archaeal genomes also contain diverse TA loci [47]. Plasmid-encoded TA systems are important for plasmid stabilization by a post-segregational killing mechanism that eliminates daughter cells that lost the TA-encoding plasmid [44]. In addition, plasmid TA systems are important for the mediation of the exclusion of co-existing compatible plasmids [41]. Chromosomally encoded TA systems typically compose parts of genomic islands [20], and it was recently suggested that they comprise important elements in the virulence of bacterial pathogens [13,41]. The objective of this work was to establish the function of the putative TA system from pUM505. We report for the first time, to our knowledge, a plasmid-encoded TA system that enhances plasmid prevalence in *Pseudomonas* cells whose toxin PumA has the potential to increase the bacterial virulence.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and plasmids

P. aeruginosa PAO1 [24] and *Escherichia coli* JM101 [45] are standard prototroph strains employed as hosts for recombinant plasmids. *E. coli* BL21-CodonPlus® (DE3)-RP (Stratagene, San Diego, CA, USA) was used for protein overexpression. PAO1-bearing plasmid pUM505 (Hg^R, Cr^R) [10] was employed to evaluate the transcriptional expression of *pumAB* genes.

The culture media used were Nutrient Broth (NB) and Luria-Bertani broth (LB); for solid media, 1.5% agar was added [18], or M9 salts minimal medium (Sigma, St. Louis, MO, USA) supplemented with 20 mM glucose and 0.1 mM CaCl₂. When necessary, Ampicillin (100 µg ml⁻¹) or Carbenicillin (400 µg ml⁻¹) was added to agar plates. Cultures were routinely grown overnight at 37 °C with shaking, and growth was monitored as Optical Density (OD) at 600 nm (OD₆₀₀) with a spectrophotometer.

The pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to recover PCR-amplified DNA fragments as well as for cloning the *pumAB* genes to assay plasmid stability. The recombinant plasmid pJET-*orf131* contains the *orf131* gene from pUM505 (accession number NC_016138; region: 119072–119269) [34], cloned in pJET1.2/blunt vector, which encodes a hypothetical protein of 65 amino acids (aa); this plasmid was employed as negative control in plasmid stability tests. The pUCP20 *Escherichia/Pseudomonas* shuttle vector [43] was used for subcloning *pum* genes to determine their participation in *Pseudomonas* virulence. pTrcHisC and pTrcHis2A vectors (Thermo Fisher Scientific, Waltham, MA, USA) were utilized for the overexpression of Pum proteins.

2.2. Genetic techniques and sequence analysis

Molecular genetics procedures were carried out by following standard methods [18]. Cloning processes were verified by DNA sequencing conducted by Elim Biopharmaceuticals, Inc. (Hayward, CA, USA). Protein sequence alignments were calculated with the CLUSTALW algorithm and BioEdit Sequence Alignment Editor Software ver. 7.2.5.0. Sequence similarities were searched in the

protein database with the BLASTP program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Probable promoter sequences were searched with the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Secondary protein structure was determined using MINNOU (Membrane protein Identification withOUT explicit) program (<http://minnou.cchmc.org/>).

2.3. RNA isolation and RT-PCR analysis

Total RNA from bacterial cells grown in LB was isolated by utilizing the Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). RNA was quantified by spectrophotometric analysis at 260 nm, previously treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Reverse Transcription-PCR (RT-PCR) was performed with total RNA samples and the Master AMP RT-PCR kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. Primers OD₁ and OR₁ (Table S1) were utilized to generate the 440-bp gene-overlapping product of the *pumAB* genes (Fig. 1a). OR₁ contains an *Xba*I restriction enzyme site (underscored) for cloning purposes (see the cloning of the Toxin–Antitoxin genes section). RT-PCR controls were treated with PCR Master Mix (Promega #M7502) and with the primer pair previously described, using genomic DNA templates (positive control) and total RNA in the absence of RT (negative control). The size of RT-PCR products was assessed by agarose (1.5%) gel electrophoresis employing the 1 kb-plus DNA ladder (Life Technologies, Rockville, MD, USA) as a marker.

2.4. Cloning of the *pumAB* genes

In order to amplify the *pumAB* genes, primers OD₂ and OR₂ (Table S1) were used, employing DNA from pUM505 plasmid as

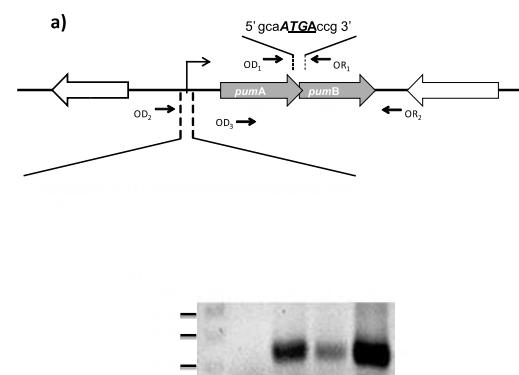


Fig. 1. Schematic representation and transcriptional analysis of *pumAB* genes from pUM505. (a) The coding regions of the *pumAB* genes are depicted by large, gray arrows indicating transcription direction; size of predicted polypeptides (aa) is also shown. Black arrows indicate positions of the oligonucleotides described in the text. Location and nucleotide sequence of the –10 and –35 regions (below) of the putative Promoter (P) are shown. The nucleotide sequences at the boundaries of the coding regions of *pumA* and *pumB* genes are depicted in the upper part. The start codon of *pumB* (bold) and the stop codon of *pumA* (underscored) are highlighted. (b) RT-PCR assays were carried out as described under Methods, and amplified fragments were separated in agarose gels. These assays used complementary DNA (cDNA) from exponential phase (lane 2) or stationary-phase (lane 3) cultures of *P. aeruginosa* PAO1 (pUM505) utilizing primers OD₁ and OR₁ for the *pumAB* intergenic region. M, Molecular size markers; PCR was performed with total RNA in the absence of RT (negative control, lane 1) and with genomic DNA templates (positive control, lane 4). An arrow to the right signals the position of the 0.44-kb amplification band.

template, and the fragment was subsequently cloned into the pJET1.2/blunt vector. In order to amplify the *pumA* and *pumB* genes independently, primers OD₂ and OR₁ and the OD₃ and OR₂ were used, respectively (Table S1) (Fig. 1a). The amplified fragments were also purified and cloned into the pJET1.2/blunt vector. Recombinant plasmids were transferred by electroporation to *E. coli* JM101 and transformants were selected on LB agar plates with Ampicillin.

For subcloning *pumAB* genes or *pum* individual genes into the pUCP20 vector, recombinant plasmids from pJET derivatives were digested with restriction enzymes *Bam*HI and *Xba*I, and DNA fragments were ligated onto the corresponding sites of pUCP20. Recombinant plasmids were transferred by electroporation to *P. aeruginosa* PAO1 or *E. coli* JM101 and the transformants were selected by culturing on LB agar with Carbenicillin or Ampicillin, respectively.

2.5. Plasmid stability tests

For stability tests, *E. coli* JM101 (pJET-*pumAB*) cultures were grown in NB without Ampicillin selection at 37 °C with shaking for 24 h. Serially diluted samples were then plated on nutrient agar with or without Ampicillin. Plates were incubated for 24 h at 37 °C and Colony-Forming Units (CFU) were measured for each condition. The initial cultures were then diluted 1:50 in fresh medium without antibiotic, incubated for 24 h, and the CFU were measured; the described procedure was repeated daily for up to 6 days. As a negative control of plasmid stability, the same procedure was followed using cultures of *E. coli* JM101 (pJET-*orf131*). To confirm the presence or absence of plasmids pJET-*pumAB* or pJET-*orf131*, CFU were obtained from nutrient agar plates with and without Ampicillin, and agarose gel electrophoresis of plasmid DNA isolated by a mini-prep procedure was performed.

2.6. Overexpression and purification of Toxin–Antitoxin proteins

The *pumA* gene coding region, as well as the DNA region from the *pumA* start codon to the *pumB* stop codon (for co-expression of both PumA and PumB proteins), were amplified by PCR using isolated pUM505 plasmid as the template; the primers employed were Dir123pTrc and Rev123pTrc or ODpTrc2A123-4 and ORpTrc2A123-4, respectively (Table S1). The amplified fragments were cloned into the pJET1.2/blunt vector, and the recombinant plasmids were transferred by electroporation to *E. coli* JM101 and transformants were selected on LB agar plates with Ampicillin. The *pumA* gene coding region was obtained after digesting the plasmid with the restriction enzymes *Pst*I and *Hind*III, and the *pumAB* region was obtained by enzyme digestion with *Bam*HI and *Hind*III; both fragments were purified and subcloned into their corresponding restriction enzyme sites of the pTrcHisC or pTrcHis2A vectors, respectively. Recombinant plasmid pTrcHis-*pumA* encodes the tagged His-PumA recombinant protein of ~16 kDa that possesses a 6X-His tag at the N-terminal end. Recombinant plasmid pTrcHis-*pumAB* encodes the PumA protein of a theoretical 11.1 kDa in size and the tagged PumB-His recombinant protein of ~17 kDa that possesses a 6X-His tag at the C-terminal end. Fragment directionality was verified by sequencing with the pTrcHis Forward primer (Thermo Fisher Scientific, Waltham, MA, USA). The recombinant plasmids were transferred by electroporation to *E. coli* BL21-CodonPlus® (DE3)-RP, followed by selection of transformants on LB agar plates with Ampicillin. Overnight cultures of *E. coli* BL21 (pTrcHis-*pumA*) or *E. coli* BL21 (pTrcHis-*pumAB*) were diluted 1:50 into 250 ml of fresh NB medium and cultured with shaking at 37 °C until OD₆₀₀ = 0.5. Overexpression of His-PumA and PumA/PumB-His (in co-expression) proteins was induced with 1 mM IsoPropyl-β-D-Thiogalactopyranoside (IPTG) and the cultures were

incubated for an additional 6 h. Cells were harvested by centrifugation at 7500 rpm for 30 min at 4 °C; the pellets were suspended in loading buffer [25 mM Tris (Tris [hydroxymethyl] amino methane)-HCl pH 6.8, 1% SDS (Sodium Dodecyl Sulfate), 0.35 mM β-mercaptoethanol, 12.5% glycerol, and 0.005% bromophenol blue] and heated for 5 min at 95 °C. Overproduction of the His-PumA and PumB-His recombinant proteins were verified by SDS-PAGE using a Tris-glycine buffer.

His-PumA and PumB-His proteins purification was performed as described by Ref. [42]. Cells were disrupted by sonic oscillation for clarification and debris was removed by centrifugation. The supernatant was loaded onto a Nickel-NTA resin (Qiagen) packed into a column. The His-PumA and PumB-His proteins were recovered by elution with 200 mM Imidazole as described by Ref. [4]. Purification was monitored by recombinant protein detection by SDS-PAGE followed by staining with Coomassie blue.

For Western blot assays, a duplicate of the previously mentioned polyacrylamide gel was transferred onto a nitrocellulose membrane (Hybond ECL). The membrane was incubated 1 h at 37 °C with HisProbe-HRP (Thermo, Rockford, IL, USA) diluted at a 1:1000 ratio, and the bound probe was visualized with the Super Signal West Pico reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Bacterial growth assays

Liquid cultures of *P. aeruginosa* PAO1 or *E. coli* JM101 containing the pUC-*pumA* recombinant plasmid were utilized to evaluate the effects of PumA protein expression. Overnight cultures were diluted 1:50 in 250 ml of fresh NB medium and growth was monitored at intervals by measuring OD₆₀₀. Serially diluted samples were then plated on nutrient agar with Ampicillin and incubated for 24 h at 37 °C and the CFU were measured.

In addition, the effects of His-PumA protein overexpression or the PumA and PumB-His proteins co-expression in *E. coli* BL21 were assayed by measuring their growth in liquid medium in the presence or absence of the IPTG inducer. Overnight cultures of *E. coli* BL21 (pTrcHis-*pumA*) or *E. coli* BL21 (pTrcHis-*pumAB*) were diluted 1:50 in 250 ml of fresh NB medium, and growth was monitored at intervals by measuring OD₆₀₀. At this point, PumA protein overexpression and PumAB proteins co-expression were induced by adding 1 mM of IPTG, and bacterial growth was monitored at intervals by measuring OD₆₀₀.

2.8. RT-qPCR assays

For *pumA* gene expression measurement from the BL21 (pTrcHis-*pumA*) strain, cultures were incubated for 6 h after their induction with 1 mM of IPTG, and cells were harvested by centrifugation. Total RNA was isolated and quantified as described in the RNA isolation method mentioned previously. Oligonucleotide primers and hydrolysis probes for RT-qPCR of the *pumA* gene and the 16S rRNA reference genes (listed in Table S1) were designed employing Biosearch Technologies software (www.biosearchtech.com/support/applications/realtimedesign-software) and were purchased from Biosearch Technologies (Novato, CA, USA). Amplification of the *pumA* gene and the 16S rRNA genes was performed using the 5' exonuclease probe RT-qPCR method. RT-qPCR was performed with total RNA samples (500 ng) and the SuperScript III Platinum One-Step RT-qPCR Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) on the LightCycler 480 II System (Roche Molecular Diagnostics, Pleasanton, CA, USA). Amplification signal curves were analyzed at absorption wavelengths of 530 nm. Appropriate positive and non-template controls were included in each test run. Relative expression of the *pumA* gene was normalized with expression values obtained from 16S rRNA genes from

P. aeruginosa (accession number AE004091; region: 722096–723631) or *E. coli* (accession number AJ605115). Estimation of relative gene expression was performed via the classical calibration dilution curve and slope calculation. A five-fold dilution series (500–0.05 ng total RNA) was prepared and utilized as sample in the RT-qPCR. Efficiency (*E*) was obtained from standard curves utilizing the formula $E = (10^{-1/\text{slope}} - 1) \times 100$. Relative expression levels were determined with the efficiency correction method, which takes into account amplification efficiencies between target and reference genes [31].

2.9. Virulence assays

2.9.1. *Caenorhabditis elegans* killing assays

C. elegans Bristol N2 [6] worms were synchronized by hypochlorite isolation of eggs from gravid adults, followed by hatching of eggs in S-basal medium [39]. L1 larvae were transferred onto NGM (Nematode Growth Medium) [39] in plates seeded with the *E. coli* OP50 strain previously grown on the plates as a food source and incubated at 20 °C for 4–5 days until they reached the young adult phase. Worms were rinsed off the plates and washed in S-basal medium. Cultures of *P. aeruginosa* strains were grown overnight in M9 broth at 37 °C, centrifuged, and the pellets were suspended in fresh M9 broth and grown to an OD₆₀₀ = 0.4. For each experiment, 20 worms were dispensed into each well of a 24-well Costar plate (Corning, Inc., Corning, NY, USA). The worms were incubated with 1×10^5 ($\pm 1 \times 10^4$) CFU of the bacterial strain contained in a total volume of 1 ml, and the plates were incubated for 36 h at 20 °C and scored for live worms at 6, 12, 24, and 36 h. *P. aeruginosa* PAO1 (pUCP20) and M9 medium were used as a positive and negative control, respectively. A worm was considered dead when it no longer responded (moved) to a touch stimulus. Worms that died as a result of becoming adhered to the wall of the plate were excluded from the analysis. For all assays, bacterial concentrations were confirmed by dilution, plating, and counting. Two independent assays with two replicates were conducted for each worm group. By supernatant effects assays the cultures of *P. aeruginosa* strains were grown overnight in M9 broth at 37 °C and centrifuged to obtain the supernatant. The worms were incubated with 100 µl of the bacterial strain supernatant in a total volume of 1 ml and the plates were incubated for 36 h at 20 °C and scored for live worms at 6, 12, 24, and 36 h. Two independent assays with two replicates per experiment were conducted for each worm group.

PumA protein toxicity on *C. elegans* was determined by His-PumA purified protein incubation. A total of 20 worms were dispensed into each well of 24-well plates (Corning) and incubated with 10, 25, 50, or 100 µg ml⁻¹ of His-PumA protein in a volume of 100 µl, and the plates were incubated for 48 h at 20 °C and scored for live worms at 6, 12, 24, 36, and 48 h. *C. elegans* was incubated in M9 medium, and M9 containing protein elution buffer with or without 0.2 M of Imidazole were used as negative controls. Additionally, His-PumA protein toxicity to *C. elegans* was analyzed after their denaturation by heating at 95 °C for 2 h or by hydrolysis utilizing 100 µg ml⁻¹ of Pronase E (Sigma-Aldrich). Finally, the PumA toxicity effect neutralized by PumB protein was determined by *C. elegans* incubation in presence of His-PumA and PumB-His purified proteins in a ratio 1:1 and 1:2. Two independent experiments with two replicates were performed for each worm group.

2.9.2. Mouse virulence experiments

The protocol for mouse manipulation assays followed the recommendations for animal manipulation issued by SAGARPA (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, México), 2001.

To assess the virulence and colonization ability of *Pseudomonas*

strains, groups of 10, 8–10-week-old male BALB/c mice weighing 18–22 g were used. The virulence assays were conducted as described by Ref. [35] and inoculated (100 µl) with as single dose administered by tail-vein injection with 3×10^6 ($\pm 3 \times 10^5$) CFU. Mouse survival was monitored each day. Two independent assays were conducted for each group.

To assess the colonization ability of *P. aeruginosa* PAO1 bearing *pumAB* genes, the mice were anesthetized with sodium pentobarbital (24 mg/kg) and inoculated via tail-vein administration with 5×10^6 ($\pm 5 \times 10^5$) CFU contained in a volume of 100 µl as described by Ref. [35]. At 48 h post-inoculation, the mice were euthanized and samples were taken for analysis. Heart, lungs, and kidneys were removed by dissection, weighed, and homogenized in 10 mM MgSO₄ utilizing a mortar and pestle. Organ homogenates were serially diluted in 10 mM MgSO₄ and plated on LB agar for CFU determination. Three independent assays were conducted for each mouse group. For all assays, bacterial inoculum concentrations were confirmed by dilution, plating, and CFU determination.

2.10. Statistical analysis

Data analysis was carried out with GraphPad Prism 5 software. Analysis Of Variance (ANOVA) employing the Tukey test was performed for all *Pseudomonas* assays. In addition, a Student *t*-test was carried out for the analysis of the expression assays. *P* < 0.05 was considered statistically significant.

3. Results and discussion

3.1. The *pumAB* genes of plasmid pUM505

Owing to their similarity with previously characterized proteins, ORF 123 and 124 from the pathogenicity island of pUM505 were reported to encode a probable TA system [34]. These ORF were renamed as *pumA* and *pumB* genes for the pUM505 Toxin–Antitoxin system. PumA is a predicted protein of 99 aa (GenBank accession number AEQ93530) that was previously reported to exhibit 55% sequence identity with the RelE protein (toxic component) of the TA system from *Klebsiella pneumoniae* (ZP_06016753) [34]. A recent BLAST analysis showed that the *pumA* gene encodes a protein that is 73% identical to an uncharacterized addition module killer protein from *Pseudomonas putida* (NCBI Reference Sequence WP_011005862). Additionally, an amino acid sequence alignment with proteins of the RelE toxin superfamily with known biological function was conducted. The alignment revealed that the PumA protein possesses only 22% identity to MjRelE from *Methanococcus jannaschii*, 18% to YoeB from *E. coli*, and 14% to PhRelE from *Pyrococcus horikoshii* (Fig. S1, Supplementary Material). The low identity of PumA with RelE-like proteins is not surprising, in that it has been reported that amino acid sequences of RelE-like proteins are quite divergent, even those originating from the same or closely related bacterial species [16]. In general, RelE-like sequences present 24–47% similarity among themselves and 15–28% identity with the canonical RelE protein from *E. coli* K-12 [15]. Although highly divergent at the primary sequence level, the PumA protein contains a secondary structure predicted by the MINNOU program that is characteristic of toxins of the RelE superfamily, constituted by five-stranded β-sheet flanked on one side by α-helices α1 and α2 and, on the other side, by α-helix α3 [15] (Fig. S1, Supplementary Material). It has been reported that, despite very low conservation at the amino acid sequence level, RelE-like proteins share secondary and tertiary structures with similar folding [16]. Additionally, PumA contains conserved-type residues (Val4, Val41, Val44, Ile53, Val61, and Val81) and highly-conserved residues (Asp40, Arg52, His54, and Arg60) that are

found in the MjRelE and YoeB proteins (Fig. S1, Supplementary Material). The mutation of Arg62 in MjRelE, which corresponds to Arg65 of PhRelE, renders a toxin inactive [15]; the Arg62 residue located in MjRelE corresponds to Arg60 in the PumA sequence. This analysis suggested that PumA could belong to the RelE toxin family.

The *pumB* gene encodes a putative transcriptional regulator of 96 aa (GenBank accession number AEQ93531) with 92% identity to the antitoxin HTH protein (Helix-Turn-Helix xenobiotic-resistant family-like proteins) from *Pseudomonas alcaligenes* (AAD40346) [34]. The PumB protein contains a predicted HTH DNA-binding domain (Fig. S2, Supplementary Material) that is characteristic of the Xre family of antitoxins, and this domain has often been found in characterized antitoxins [44]. In type II TA systems, the antitoxin is generally a DNA-binding protein that binds to the operator of the operon to repress its own transcription. In the majority of cases, the HTH motif is located at the N-terminal domain of the antitoxins [11]. Examples of the existence of the HTH motif in solved structures of antitoxins include PezA [21], as well as Higa [37]. However, the HTH motif of PumB was located at the C-terminal region of the protein (Fig. S2, Supplementary Material). A similar position of the HTH motif was found in the functional antitoxin MqsA of *E. coli* [8]. This analysis suggests that the PumB protein could function as an HTH-type antitoxin protein.

As mentioned previously, the *pumA* gene encodes a putative RelE-type toxin, while the *pumB* gene encodes a putative HTH-type antitoxin, and it is suggested that these genes encode a hybrid TA system. In general, it has been assumed that each toxin family is associated with a specific antitoxin family; for instance, RelE proteins are frequently associated with RelB antitoxins [17]. However, many hybrid systems exist in which a TA locus contains a toxin of one class and an antitoxin of another class [3]. Together, these results suggest that *pumAB* genes from plasmid pUM505 encode a putative TA hybrid system.

3.2. *pumAB* genes form an operon

The *pumA* and *pumB* genes exhibited a genetic arrangement where *pumA*, which encodes a putative toxin, was located upstream of *pumB*, which encodes an antitoxin (Fig. 1a). In the majority of TA systems, antitoxin genes are located upstream of their cognate toxin genes, suggesting that antitoxins appear to possess an advantage over their cognate toxins with regard to their production [44]. However, there are exceptional cases in which toxin genes are located upstream of their cognate antitoxin genes, suggesting that these differences in gene order might exert a subtle effect on the toxin-to-antitoxin ratio in the cell, which in turn regulates toxin function [44]. Additionally, sequence analysis revealed that *pumA* and *pumB* genes contain a putative σ^{70} promoter that showed at the non-typical –35 to –10 box in the 5' region of *pumA* (Fig. 1a); no promoter region was identified for *pumB*, suggesting that these two genes constitute an operon that could be expressed from the *pumA* promoter. In type II TA systems, toxin and antitoxin genes are commonly co-transcribed, forming operons [13], suggesting an evolutionary advantage of the close vicinity of coding regions. Additionally, *pumAB* genes have overlapped coding regions; the stop codon of *pumA* and the initiation codon of *pumB* share four nucleotides (Fig. 1a). Overlapping of coding sequences is a conserved feature of paired TA systems [23].

To investigate the existence of a possible transcriptional linkage of the *pumAB* genes, RT-PCR analyses were performed. Primers were designed to amplify complementary DNA (cDNA) synthesized from a transcript spanning the overlapping *pumAB* region (Fig. 1a). When total RNA from exponential-phase cultures of the *P. aeruginosa* PAO1 (pUM505) strain was probed, a sole ~440-bp DNA fragment, covering the overlapping region, was detected

(Fig. 1b, lane 2). No corresponding fragment was detected in the negative control (Fig. 1b, lane 1). A less intense DNA band was observed with RNA obtained from stationary-phase cultures (Fig. 1b, lane 3), which is in agreement with previous reports of TA systems displaying higher expression levels in the exponential growth phase [26]. These data demonstrate that the *pumAB* genes are co-transcribed into a bicistronic mRNA, and strongly suggest that they form an operon.

3.3. The *pumAB* genes confer plasmid stability

Since the function of plasmid-encoded TA systems is related to plasmid stabilization, we investigated whether *pumAB* genes are able to promote plasmid maintenance. Because pUM505 additionally to possess the putative TA system contains several genes probably related with the partition, segregation, maintenance, and stabilization of plasmids, as well as *parA*, *parB*, *xerD*, *xerC*, and *ssb*, respectively, [34], in order to test this, *pumAB* genes were first cloned into the Ampicillin (Ap)-resistance pJET vector and the recombinant plasmid pJET-*pumAB* obtained was transferred to the *E. coli* JM101 strain. Plasmid segregation was tested by measuring CFU in *E. coli* JM101 (pJET-*pumAB*) and JM101 (pJET-*orf131*) strains after 24-h consecutive subcultures in non-selective NB medium; samples were withdrawn each day, diluted, and plated on nutrient agar with or without Ap, as described under Methods. CFU of pJET-*orf131*-containing cells decreased in each subculture on the Ap plates from day 3 (corresponding to 216 generations) and, after day 6 (corresponding to 432 generations), these were undetectable (Fig. 2). In contrast, CFU counts in transformants bearing the pJET-*pumAB* plasmid remained without significant changes throughout the assay. Agarose gel electrophoresis of mini-prep plasmid DNA isolations from the subcultures confirmed the absence of plasmid bands from pJET-*orf131* clones, as well as the presence of recombinant plasmid in clones from pJET-*pumAB* transformants (Fig. S3, Supplementary Material). These data indicate that the presence of *pumAB* increases the stability of the pJET plasmid and suggest that they do so by means of a post-segregation killing mechanism

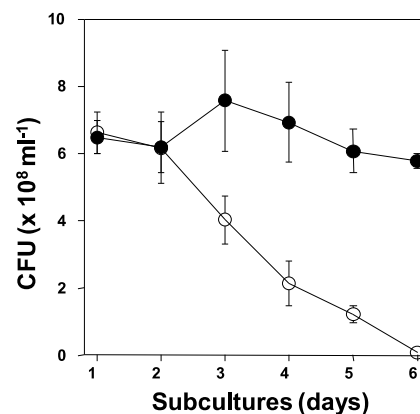


Fig. 2. Plasmid stability conferred by *pumAB* genes from pUM505. Cultures were grown in Nutrient Broth at 37 °C with shaking, and consecutive 24-h subcultures were performed without antibiotic selection. Serial dilutions from each subculture were plated on Nutrient agar medium with Ampicillin, and CFU were counted as described under Methods. Symbols: (○), *E. coli* JM101 (pJET-*orf131*) as a negative control; (●), *E. coli* JM101 (pJET-*pumAB*). Data shown are the means of three independent assays in duplicate with Standard Error (SE) bars shown.

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3.4. Overexpression of the *pumA* gene inhibits *E. coli* growth

To investigate whether a functional toxin is encoded by *pumA* of pUM505, this gene, including its putative promoter, was subcloned into the pUCP20 shuttle vector; recombinant plasmid pUC-*pumA* was transferred to the *P. aeruginosa* PAO1 strain, and growth kinetics in liquid cultures was measured. The presence of the pUC-*pumA* plasmid did not cause growth inhibition in PAO1 cells (Fig. S4a, Supplemental Material). This effect was not expected because the *pumA* gene was identified in a plasmid from a *Pseudomonas* strain and, as was shown, is expressed in this strain together the *pumB* gene as an operon (Fig. 1b). However, an explanation to this result may be attributed to the fact that the *pumA* gene is not expressed in PAO1 under its own promoter into the pUC20 vector. However, another possibility could be that the product encoded by the *pumA* gene was less toxic to PAO1 than to *E. coli*, bearing in mind that the function of *pumAB* genes in plasmid stability was determined in *E. coli*. To discern between these possibilities, *pumA* gene expression was determined by RT-qPCR assays from cultures of *P. aeruginosa* PAO1 containing the recombinant pUC-*pumA* plasmid. The results showed that the *pumA* gene was expressed in the *P. aeruginosa* PAO1 strain (Fig. S4c, Supplemental Material). The latter suggested that this gene is functional in this strain. To determine whether the product of the *pumA* gene affects the growth of *E. coli* cells, recombinant plasmid pUC-*pumA* was transferred to *E. coli* JM101 and growth kinetics in liquid cultures were analyzed. The results demonstrated no differences in the growth of *E. coli* cells through the presence of the pUC-*pumA* plasmid (Fig. S4b, Supplemental Material). Additionally, the results of expression by RT-qPCR analysis showed that the *pumA* gene was expressed in this strain (Fig. S4c, Supplemental Material). These results noted that when PumA is expressed in physiological levels, it does not affect cell growth, suggesting that a high expression level is required. In *Mycobacterium tuberculosis*, a correlation between the expression levels of the VapC toxin from TA systems and its ability to confer a toxic phenotype has been reported [2]. To determine whether the overexpression of PumA affects bacterial growth, the *pumA* gene was subcloned into the pTrcHisC expression vector under the control of an IPTG-inducible promoter, and recombinant plasmid pTrcHis-*pumA* was transferred to the *E. coli* BL21 strain. Growth kinetics with or without IPTG induction was then measured in BL21 derivatives. BL21 cells containing the vector alone, with IPTG, exhibited no harmful effects on growth. In contrast, IPTG-induced BL21 transformants containing recombinant plasmid pTrcHis-*pumA* displayed clear growth inhibition as compared with non-induced transformants (Fig. 3a), and these results were consistent with a decrease in the CFU count (Fig. S5, Supplemental Material). Additionally, *pumA* gene expression levels with or without IPTG induction were determined, and the results showed an increase of about 70 times of the *pumA* transcript with respect to the non-induced condition (Fig. 3b), indicating that overexpression of PumA affects *E. coli* growth. Several reports demonstrated that overexpression of the toxins of TA systems produced cell growth inhibition; for example, in *E. coli* K-12 (MG1655), RelE, MazF, YeeV, and YkfI toxins inhibited bacterial growth when they were ectopically overexpressed [7,30]. Additionally, has been reported that some toxins caused cell growth inhibition when they were heterologously and ectopically overexpressed in *E. coli* BL21, as well as the VapC-3 and Tox28 toxins from *Acidithiobacillus ferrooxidans*, YoeB from *Streptococcus suis*, and YoeB from *Staphylococcus equorum* [9,26,46]. Therefore, our results indicated that, like others toxins from TA systems, a high expression

level of the *pumA* gene is required to affect bacterial growth, suggesting that PumA functions as a toxin in *E. coli*. Since many genes can be toxic to cells when they are overexpressed, it was important to determine whether cell growth inhibition by PumA is neutralized by expressing its cognate antitoxin. We compared the growth of *E. coli* BL21, in which the *pumA* was expressed either individually or together with its cognate *pumB*, under the control of the same inducible promoter, to determine whether *pumB* allowed cell growth in the presence of the inducer. The *pumAB* gene pair was cloned into the pTrcHis2A vector and transferred to *E. coli* BL21, as described in Methods. When co-expression of PumA and PumB-His proteins was induced by IPTG, BL21 cells did not exhibit growth inhibition in comparison with the strain with individual His-PumA overexpression (Fig. 3a). These results were consistent with the CFU count (Fig. S5, Supplemental Material), suggesting that PumB protein could neutralize the growth inhibitory effect of PumA. Overexpression of recombinant His-PumA and PumB-His proteins was verified by SDS-PAGE and by Western blot analysis (Fig. S6, Supplementary Material). In type II TA systems, the major role of the antitoxin is to neutralize the toxic function of its cognate toxin mediated by the toxin-binding domain [44]. The majority of the TA systems from *E. coli* and *Mycobacterium smegmatis* tested experimentally showed that the toxin inhibited bacterial growth, indicating an effect that is neutralized by its cognate antitoxin [36]. These results indicate that PumA acts as a toxic protein in *E. coli* and that its effects are neutralized by the presence of PumB protein, which supports the notion that these proteins form a functional TA system in *Pseudomonas*.

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It has been reported that some strains of *P. aeruginosa*, such as the highly virulent PA14 strain, can kill the *C. elegans* nematode, utilizing virulence factors that are also required for pathogenesis in mammalian or plant hosts [40]. In addition, chromosomally encoded TA systems may be related to the virulence of bacterial pathogens [13,41]. To determine whether the *pumAB* TA system from pUM505 participates in *P. aeruginosa* virulence, assays to test their ability to kill *C. elegans* were conducted. Since the pUM505 contains several genes that are related with the virulence of *P. aeruginosa* [35], *pumAB* genes were cloned, either paired or individually, in the pUCP20 vector, and recombinant plasmids pUC-*pumAB*, pUC-*pumA* and pUC-*pumB* were transferred to *P. aeruginosa* PAO1. The results revealed that transformants containing the *pumAB* genes displayed similar viability levels as compared to the vector-only PAO1 control strain (~60% of worms killed in 36 h) (Fig. 4a), suggested that in the conditions carried out the PumAB TA system is not involved in the virulence of *P. aeruginosa*. However, interestingly transformants with the *pumA* gene in the same conditions assayed increased PAO1 ability to kill worms at ~95% at 36-h post-incubation as compared to the control strain, whereas transformants with plasmid constructions containing *pumB* displayed similar viability levels as compared to the control strain (~60% of worms killed in 36 h) (Fig. 4a). In order to rule out the possibility that the virulence effects observed may be due to differences in the growth rate of the transformants analyzed, growth assays were performed and the results exhibited no harmful effects on growth among the transformants (Fig. S7, Supplementary Material). These data indicate that the *pumA* gene product improves the ability of PAO1 to kill *C. elegans*, this effect is neutralized by the *pumB* gene product. Additionally, the effect of the supernatant from *Pseudomonas* strains on the *C. elegans* viability was assayed and the results showed that the supernatant from *P. aeruginosa* contained the *pumA* gene was able to kill the ~75% of worms in 36 h of incubation;

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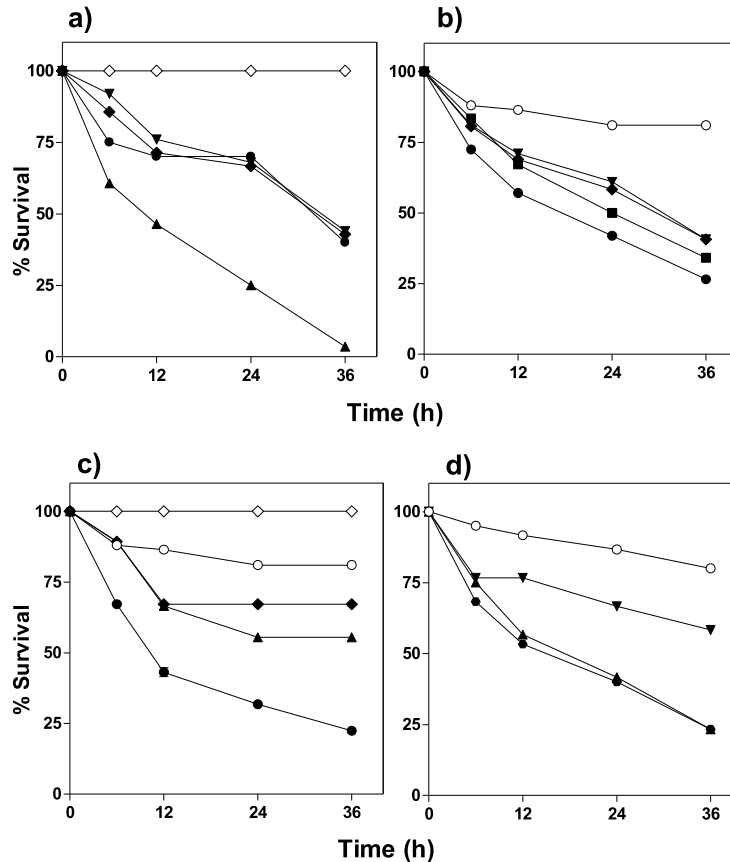


Fig. 4. The product of *pumA* gene kills *Caenorhabditis elegans*. (a) Effects of *P. aeruginosa* transformants with *pumAB* genes on *C. elegans* viability. Groups of 20 worms were incubated with $1 \times 10^5 (\pm 1 \times 10^4)$ Colony-Forming Units (CFU) of the indicated strains, as described under Methods. Mixtures were incubated at 20°C and scored for live worms at the indicated times. Symbols: ($\diamond$), M9 medium; ($\bullet$), *P. aeruginosa* PAO1 pUCP20; ($\blacklozenge$), *P. aeruginosa* PAO1 (pUC-*pumA*); ($\blacktriangle$), *P. aeruginosa* PAO1 (pUC-*pumB*). (b) Effects of the PumA protein on *C. elegans* viability. Groups of 20 worms were incubated with purified His-PumA as described under Methods. Mixtures were incubated at 20°C and scored for live worms at the indicated times. Symbols: ($\diamond$), M9 medium with 0.2 M Imidazole; ($\blacktriangledown$), ($\blacklozenge$), ($\blacksquare$), and ($\bullet$), M9 medium with 10, 25, 50, or 100 $\mu\text{g ml}^{-1}$ of His-PumA, respectively. (c) Effects of previously denatured His-PumA on *C. elegans* viability. Symbols: ($\diamond$), M9 medium; ($\diamond$), M9 medium with 0.2 M Imidazole; ($\bullet$), M9 medium with 100 $\mu\text{g ml}^{-1}$ of His-PumA; ($\blacktriangle$), and ($\blacklozenge$), M9 medium with 100 $\mu\text{g ml}^{-1}$ of His-PumA denatured by Pronase E or by heat, respectively. (d) Neutralization of the PumA protein effects on *C. elegans* viability by PumB. Groups of 20 worms were incubated with purified His-PumA with or without PumB as described under Methods. Mixtures were incubated at 20°C and scored for live worms at the indicated times. Symbols: ($\diamond$), M9 medium with 0.2 M Imidazole; ($\bullet$), M9 medium with 5 $\mu\text{g ml}^{-1}$ of His-PumA; ($\blacktriangle$) and ($\blacktriangledown$), M9 medium with 5 $\mu\text{g ml}^{-1}$ of His-PumA with 1:1 or 1:2 (His-PumA:PumB-His) ratio, respectively. The data shown are means from the duplicates of two independent assays.

the virulence in an animal model, a total of $3 \times 10^6 (\pm 3 \times 10^5)$ CFU of PAO1-derivative strains were individually inoculated by intravenous (i.v.) administration, and mouse survival was monitored each day. It was found that mice infected with *P. aeruginosa* PAO1 (pUC-*pumA*) led to 20% lethality at day 3 post-inoculation, and reached 60% lethality at day 12 compared with mice inoculated with *P. aeruginosa* (pUCP20) or with *P. aeruginosa* (pUC-*pumB*), which both maintained 100% survival until day 12 post-inoculation (Fig. 5a). This result indicates that the toxin encode by the *pumA* gene increase the pathogenicity of *P. aeruginosa* in a mammalian host. Additionally, the effect of the *pumA* on mouse colonization was determined with the previously mentioned PAO1-derivative strains, as described under Methods. The presence of the *pumA* gene significantly increased PAO1 CFU in mouse heart (two-fold),

lung (six-fold), and kidney (six-fold) as compared with those inoculated with transformants with the plasmid containing the *pumB* gene, which yielded CFU counts similar to those of the empty-vector strain (Fig. 5b). To determine whether the expression of the PumB antitoxin could neutralize the virulence effects supplied by the presence of the *pumA* gene, mice were inoculated with *P. aeruginosa* transformants containing *pumAB* genes. The results showed that the CFU count was similar to that of the empty-vector strain or to that of the plasmid containing the *pumB* gene (Fig. 5b). These data indicate that PumA expressed alone possess the ability to increases PAO1 invasiveness into mouse organs, an event neutralized by the *pumB* product. Several chromosomal TA systems have been shown to cause increased virulence in their bacterial hosts against animals, including TA systems from *E. coli* [27],

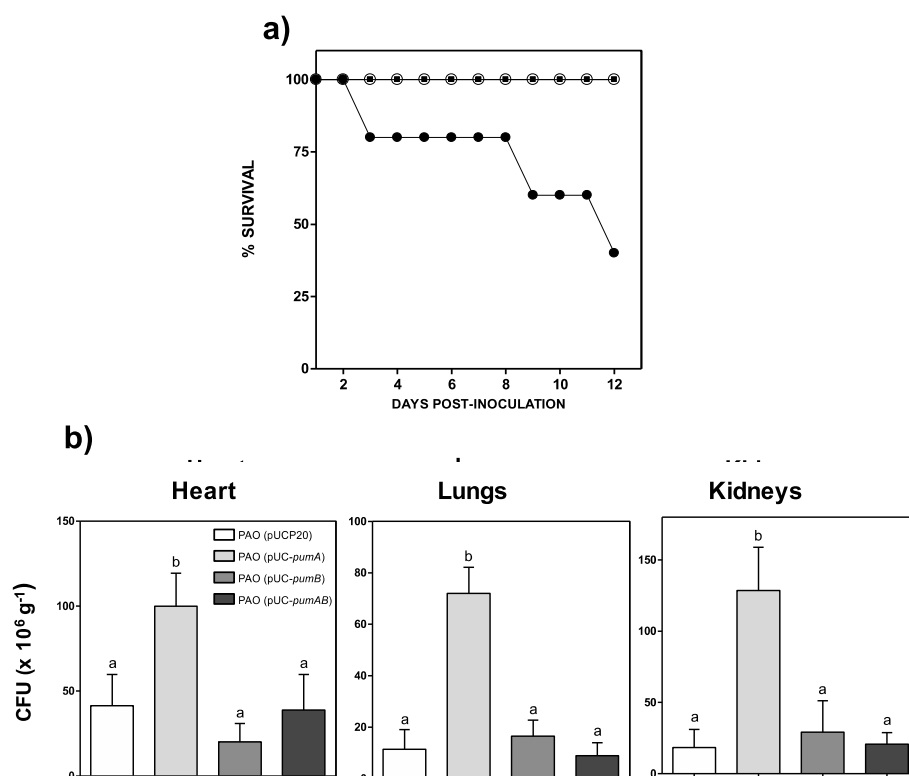


Fig. 5. Mouse virulence by *Pseudomonas aeruginosa* strains. (a) Bacterial virulence in mice was determined performing inoculations with a total of $3 \times 10^6 (\pm 3 \times 10^5)$ Colony-Forming Units (CFU) by intravenous (i.v.) administration. Mouse survival was monitored daily for 12 days. Symbols, mice inoculated with: (○), *P. aeruginosa* PAO1 (pUCP20); (■) *P. aeruginosa* PAO1 (pUC-pumB), and (●), *P. aeruginosa* PAO1 (pUC-pumA). Plots show the mean data pulled from two independent experiments expressed as percentages of mice surviving post-infection in each experimental group. (b) Mouse invasivity by *P. aeruginosa* strains. Groups of 10, 8–10-week-old male BALB/c mice were infected with $5 \times 10^6 (\pm 5 \times 10^5)$ CFU of the indicated strains, as described under Methods. At 48 h post-inoculation, heart, lungs, and kidneys were removed by dissection and their homogenates were serially diluted and plated on Luria Broth agar for determination of CFU counts. Control corresponds to the PAO1 pUCP20 strain. Values are the means of three independent experiments conducted for each mouse group. One-way ANalysis OF VAriance (ANOVA) with Tukey post-hoc test was used to compare the strains, and significant differences ($p < 0.05$) are indicated with the letters a and b.

Salmonella typhimurium [13], *M. tuberculosis* [36], *L. interrogans* [22] however, this relationship has not been reported for elements of plasmid-encoded TA systems. These results further confirmed that *pumAB* genes encode a TA system, comprising the PumA toxin and its cognate PumB antitoxin, being the toxin the element with capacity to generate toxic effects. To our knowledge, this is the first toxin from a plasmid-encoded TA system from *P. aeruginosa* that has demonstrated participation in virulence. The increase of PAO1 virulence and the invasion by PumA toward the organisms employed in this work (invertebrates and mammals) suggest that the virulence mechanism involved may be conserved in these evolutionarily divergent hosts. The mechanism by which the PumA toxin exerts its toxic actions is currently under study.

In conclusion, pUM505 contains *pumAB* that encodes a TA system conferring plasmid stability and encodes a toxin with potential to increasing *P. aeruginosa* virulence in animal models.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.micpath.2017.09.060>.

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Supplementary material

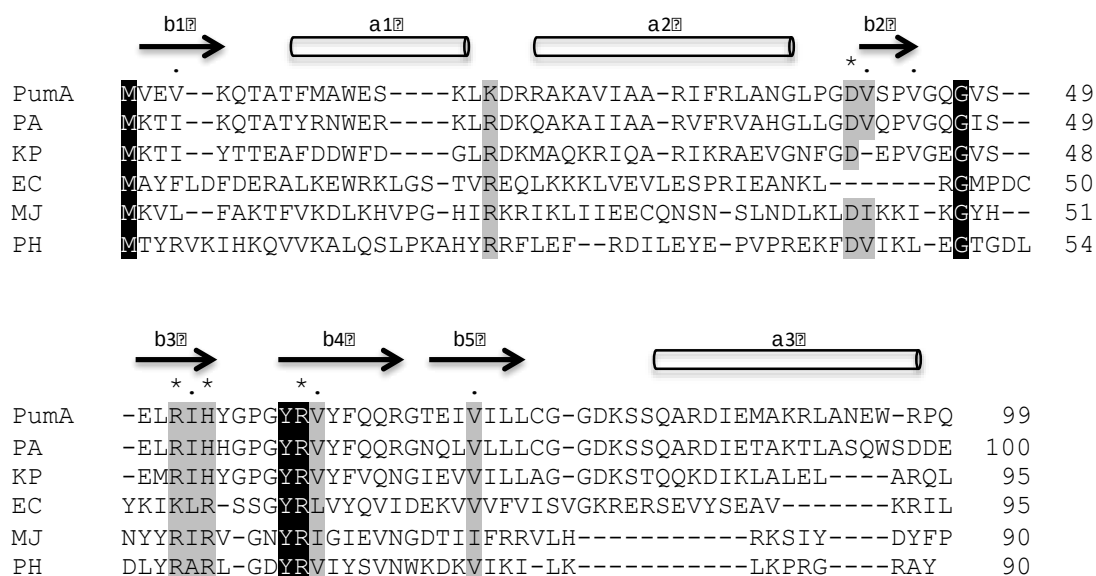


Figure S1. Multiple sequence alignment of RelE homologues. Shaded black and grey areas correspond to identical and similar residues, respectively. Conserved residues among RelE family members are indicated by asterisks, and dots denote type-conserved residues. Secondary-structure elements were predicted using the MINNOU program according to PumA protein from pUM505 are indicated above the sequences. RelE proteins: PumA, putative RelE-type toxin from pUM505; PA, putative toxin from *Pseudomonas putida*; KP, putative RelE from *Klebsiella pneumoniae*; EC, YoeB (RelE-type toxin) from *Escherichia coli*; MJ, MjRelE (RelE-type toxin) from *Methanococcus jannaschii*, and PH, PhRelE (RelE-type toxin) from *Pyrococcus horikoshii*.

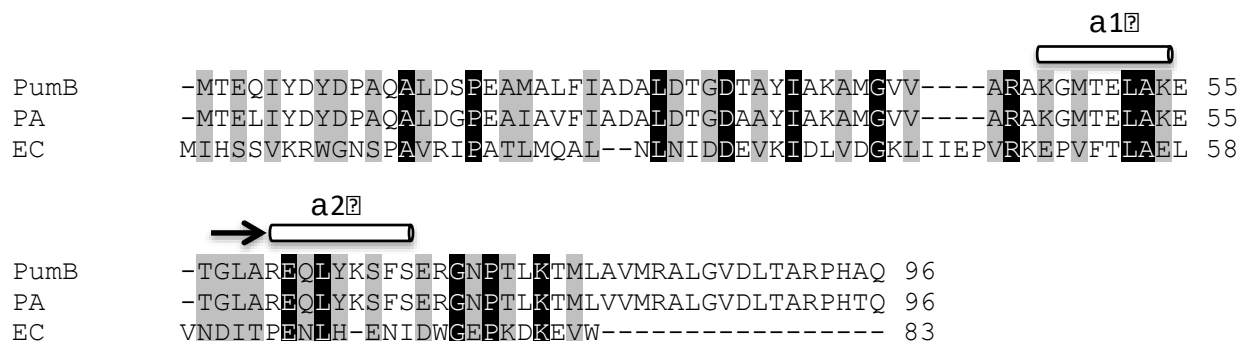


Figure S2. Multiple sequence alignment of HTH-type antitoxin homologues. Shaded black and grey areas correspond to identical and similar residues, respectively.

Secondary-structure elements predicted using the MINNOU program corresponding to the Helix-Turn-Helix (HTH) DNA-binding domain according to PumB from plasmid pUM505 are indicated above the sequences. HTH-type proteins: PumB, putative antitoxin from pUM505; PA, HTH putative protein from *Pseudomonas alcaligenes* and EC, MazE antitoxin from *E. coli*.

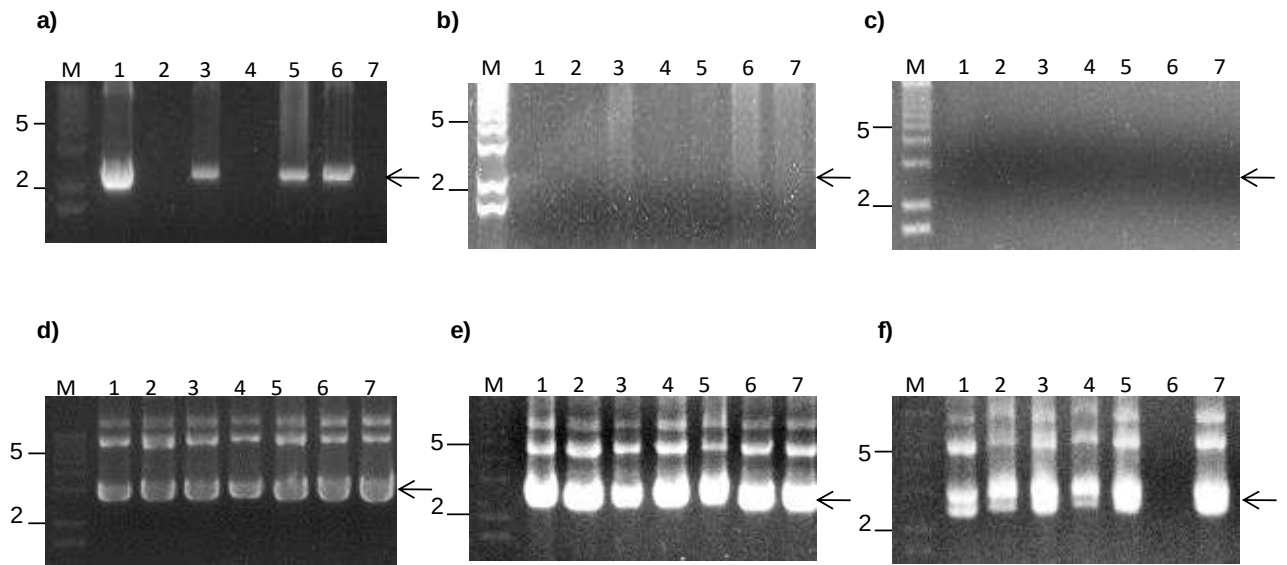


Figure S3. Isolation of plasmids from *E. coli* JM101 transformants. Agarose gels show the presence or absence of plasmid bands of pJET-*orf131* (**a, b, c**) or pJET-*pumAB* (**d, e, f**) from colonies obtained from nutrient agar plates without Ampicillin. M, Molecular size markers (in kilobases); lanes 1–7, plasmid DNA mini-prep isolation of independent samples from day 2 (**a, d**), day 4 (**b, e**), and day 6 (**c, f**) subcultures. Arrows to the right signal the position of pJET-derivative plasmids.

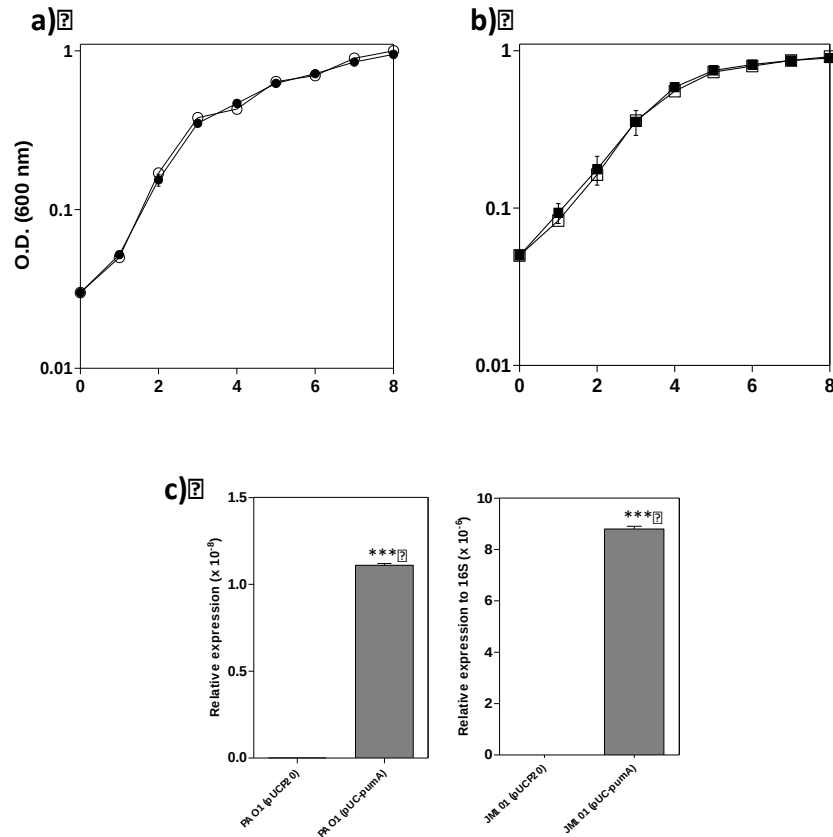


Figure S4. Effects of *pumA* gene expression on bacterial growth. (a) Growth of *P. aeruginosa* PAO1 or (b) *E. coli* JM101 strains with or without the *pumA* gene. Bacterial cultures were grown overnight in LB broth and diluted 1:100 in 25 ml of fresh medium. Flasks were incubated at 37°C with shaking, and growth was monitored at intervals as Optical Density (OD) at 600 nm (OD₆₀₀) with a spectrophotometer. (O), *P. aeruginosa* PAO1 (pUCP20); (●), PAO1 (pUC-*pumA*); (□), JM101 (pUC20), and (■), JM101 (pUC-*pumA*). Data shown are the means of three independent assays, in duplicate with Standard Error (SE) bars shown. (c) Expression levels of *pumA* from cultures of *P. aeruginosa* PAO1 (left) or *E. coli* (right) strains with or without the pUC-*pumA* plasmid determined by RT-qPCR. Values represent the means of two independent determinations in duplicate with Standard Error (SE) bars shown, normalized with respect to transcription of the 16S gene from *P. aeruginosa* or *E. coli*, respectively. Statistical analysis was conducted with Student *t* test to compare the expression and significant differences ($p < 0.0001$) and are indicated by asterisks (***)

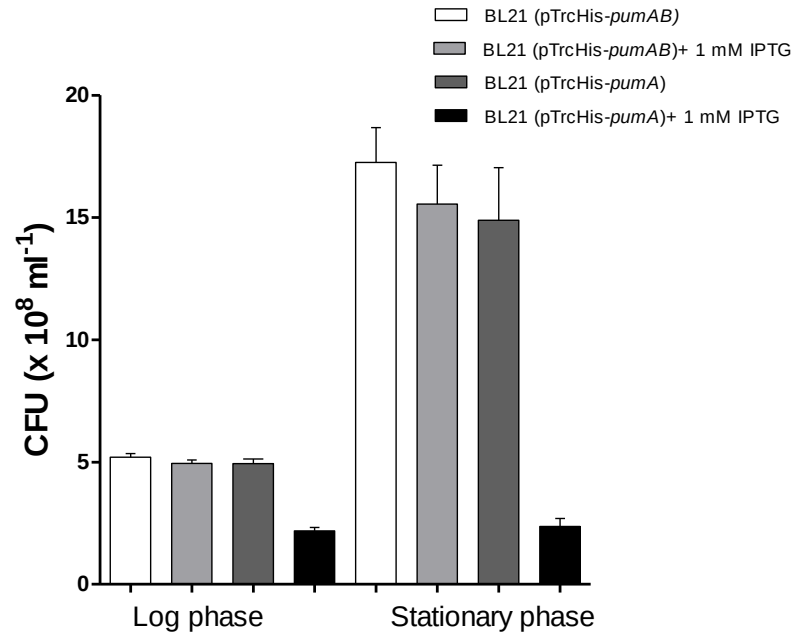


Figure S5. Viability of *E. coli* cells by overexpression of *pumA* or *pumAB* co-expression. Cultures of *E. coli* BL21 (pTrcHis-*pumA*) or *E. coli* BL21 (pTrcHis-*pumAB*) were grown at 37°C with shaking in Nutrient Broth, and growth was monitored at intervals at 600 nm (OD₆₀₀) with a spectrophotometer. Serially diluted samples from log and stationary phase were taken and plated on nutrient agar with Ampicillin. Plates were incubated for 24 h at 37°C and Colony-Forming Units (CFU) were measured for each condition. The data shown are means from the duplicates of two independent assays with Standard Error (SE) bars shown.

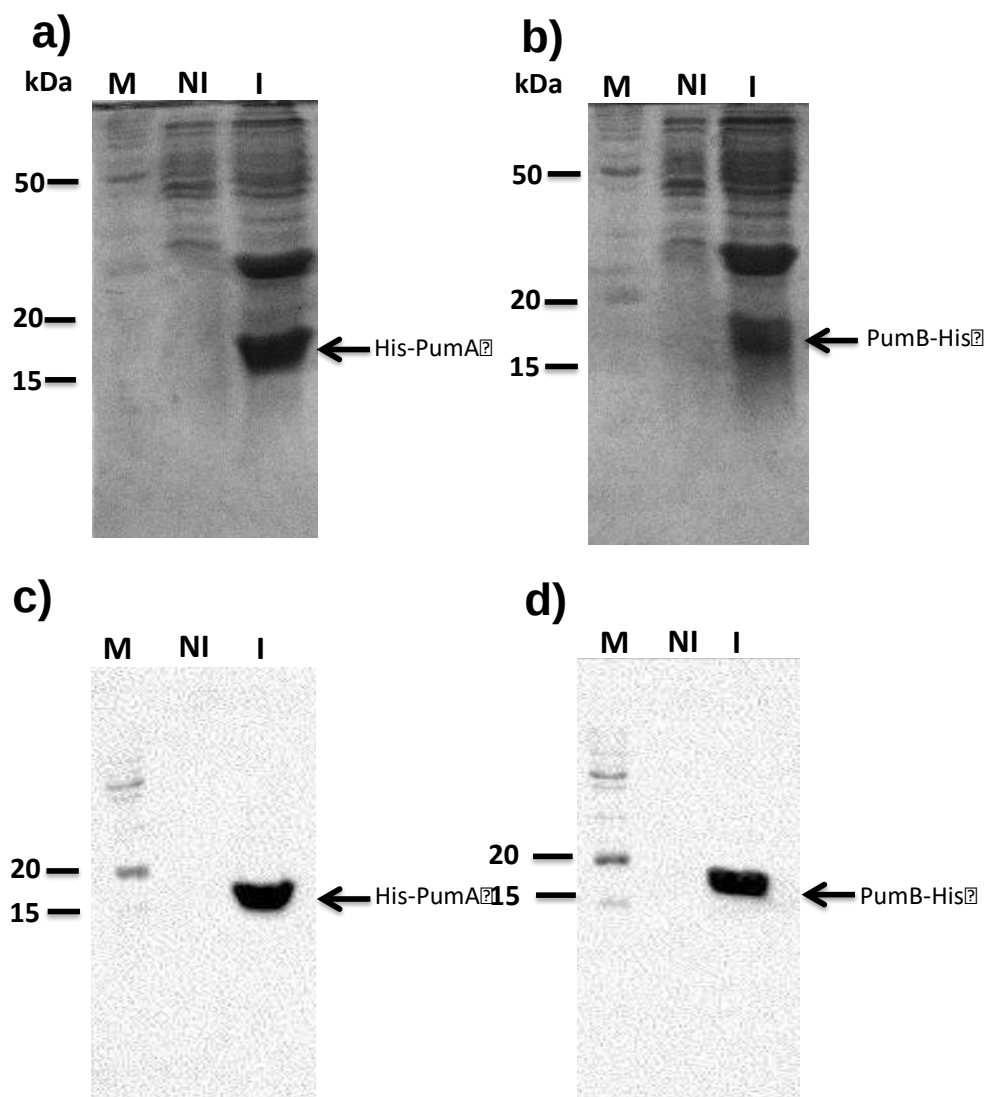


Figure S6. Overexpression and detection of recombinant His-PumA and PumB-His proteins. SDS-PAGE analysis of lysates from *E. coli* BL21-Codon-Plus (DE3)-RP cells carrying pTrcHis-*pumA* **(a)** or pTrcHis-*pumAB* **(b)** plasmids Not Induced (NI) or Induced (I) with 1 mM IPTG. M, Molecular mass markers shown to the left in kDa. Immunoblot of His-PumA **(c)** or PumB-His **(d)** protein detection using HRP. Arrows to the right signal the position of the recombinant proteins.

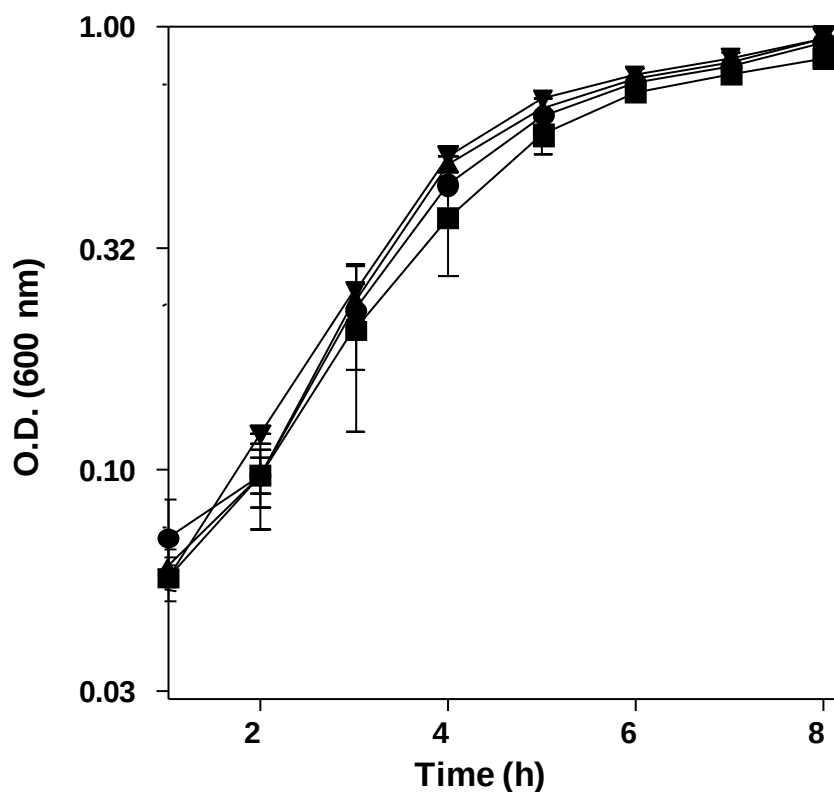


Figure S7. Growth kinetics of *P. aeruginosa* strains. Bacterial cultures were grown overnight in LB broth and diluted 1:100 in 25 ml of fresh medium. Flasks were incubated at 37°C with shaking, and growth was monitored at intervals as Optical Density (OD) at 600 nm (OD₆₀₀) with a spectrophotometer. (●), *P. aeruginosa* PAO1 pUCP20; (■), PAO1 pUC-*pumA*; (▲), PAO1 pUC-*pumB*, and (▼), PAO1 pUC-*pumAB*. Data shown are the means of three independent assays in duplicate with Standard Error (SE) bars shown.

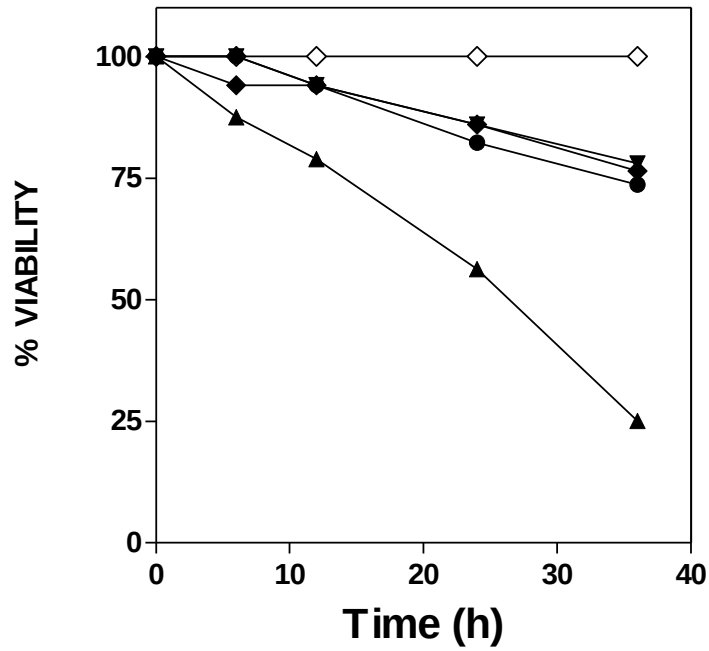


Figure S8. Effects of supernatants from *P. aeruginosa* strains on the *Caenorhabditis elegans* survival. Groups of 20 worms were incubated with 100 μ l of the supernatant obtained from a culture of *P. aeruginosa* strains, as described under Methods. Mixtures were incubated at 20°C and scored for live worms at the indicated times. Symbols: (◇), M9 medium; (●), *P. aeruginosa* PAO1 pUCP20; (◆), *P. aeruginosa* PAO1 (pUC-*pumAB*); (▲), *P. aeruginosa* PAO1 (pUC-*pumA*), and (▼), *P. aeruginosa* PAO1 (pUC-*pumB*). The data shown are means from the duplicates of two independent assays.

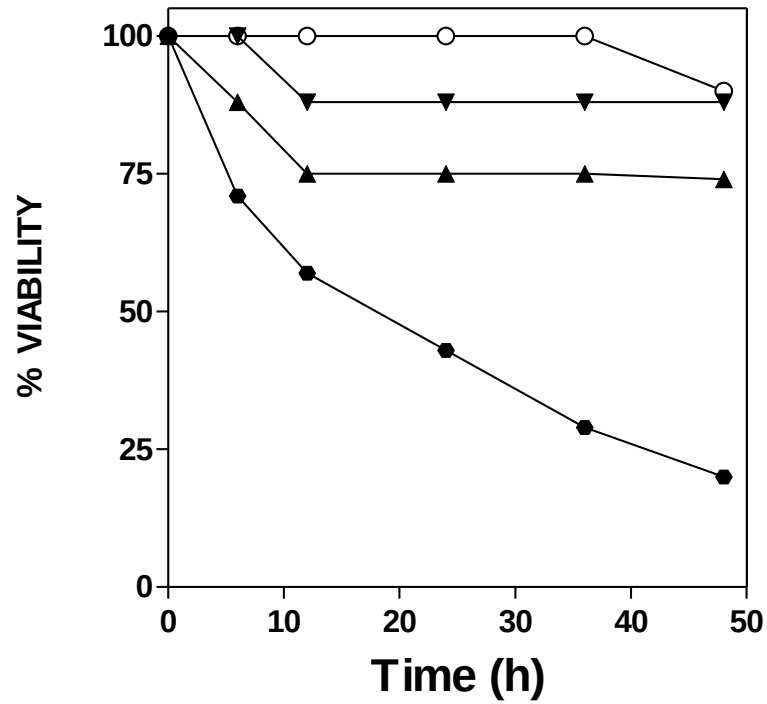


Figure S9. Effects of buffers employed for protein elution on the *Caenorhabditis elegans* survival. Groups of 20 worms were incubated with M9 medium (○), M9 with 100 µg ml⁻¹ of His-PumA protein (●), M9 with buffer of protein elution without Imidazole (▼), and M9 with buffer of protein elution with 0.2 M Imidazole (▲). Mixtures were incubated at 20°C and scored for live worms at the indicated times. The data shown are means from the duplicates of two independent assays.

CAPITULO II

Artículo: Role of PumB Antitoxin as a Transcriptional Regulator of the PumAB Type-II Toxin-Antitoxin System and its Endoribonuclease Activity on PumA (Toxin) transcript.

**Role of PumB Antitoxin as a Transcriptional Regulator of the PumAB Type-II
Toxin–Antitoxin System and its Endoribonuclease Activity on PumA (Toxin)
Transcript**

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Abstract

Toxin–antitoxin (TA) systems are important in bacterial survival because they regulate cell growth and death under stressful conditions. PumAB type-II TA system, encoded by the pUM505 plasmid, confers plasmid stability on *Escherichia coli*. In addition, the PumA toxin possess the capability to increases the virulence of *Pseudomonas aeruginosa*, an effect that is neutralized by the PumB antitoxin. The aim of this study was to establish the mechanism of regulation of the PumAB system from pUM505. By an *in silico* analysis of the putative regulatory elements we identify two putative internal promoters P_{pumB} and $P_{pumB-AlgU}$ (in addition to the already reported P_{pumAB}) located upstream of *pumB*. By qRT-PCR assays, we determined that the *pumAB* genes are transcribed differentially, since the mRNA of *pumB* is more abundant than the *pumA* transcript. We also observed that *pumB* could be expressed individually and its mRNA levels decreased under oxidative stress, during individual expression as well as co-expression of *pumAB*. *pumA* mRNA levels; however, were not affected. This suggests negative regulation of *pumB* by stressful conditions. The PumB purified protein was bound to a DNA region located between the P_{pumAB} and the *pumA* coding region, and PumA improved its binding ability, suggesting that a PumA-PumB complex co-regulates the transcription of *pumAB* operon. Interestingly, the *pumA* mRNA levels decreased on incubation *in vitro* with PumB protein. This effect was repressed by ribonuclease inhibitors, suggesting that PumB could possess endoribonuclease activity towards the mRNA of the toxin. Taken together, we conclude that the PumAB TA system possesses multiple mechanisms to regulate its expression as well as the mRNA toxin levels, and our

42 analyses provide new insights into the understanding of the control of TA systems
43 from mobile plasmid-encoded genes from a human pathogen.

44 **Key words:** PumAB toxin-antitoxin system, transcriptional regulator,
45 Endoribonuclease activity, *Pseudomonas aeruginosa*.

46

Introduction

Toxin-antitoxin (TA) systems are modules found in bacterial mobile genetic elements and in bacterial chromosomes (Leplae et al. 2011; Page and Peti 2016). Plasmid-encoded TA systems are responsible for plasmid stabilization by post-segregational killing mechanism (Yamaguchi et al. 2011), and the chromosomally encoded TA systems have been associated with the virulence of bacterial pathogens (Hayes and Van Melder 2011; Unterholzner et al. 2013; De la Cruz et al. 2013). The PumAB TA system encoded by the conjugative plasmid pUM505, isolated by a clinical strain of *Pseudomonas aeruginosa*, confers plasmid stability on *Escherichia coli* (Hernández-Ramírez et al. 2017). The PumA toxin inhibits bacterial growth when it is overexpressed, while its toxic effects are neutralized by the PumB antitoxin. Interestingly, the PumA toxin also increases the virulence of *P. aeruginosa*, indicating that PumA is a potential virulence factor for the bacteria (Hernández-Ramírez et al. 2017); suggesting that PumAB it is not a conventional TA system at display both plasmid-stability and virulence functions. In this study was established the mechanism of regulation of the PumAB system from pUM505.

In general, the TA systems are encoded by an operon, where the antitoxin gene usually precedes the toxin gene. This arrangement contributes to a high antitoxin to toxin ratio and ensures that the toxin is inactivated under non-stress conditions (reviewed in Sofos et al. 2015). In contrast with most of the TA systems, the *pumAB* operon displays a different genetic arrangement, in which the *pumA* gene (toxin) is located upstream of the *pumB* gene (antitoxin) (Hernández-Ramírez et al. 2017). Some exceptional cases of TA systems in which toxin genes are located upstream

of their cognate antitoxin genes had been described. In these cases, such differences in gene order might exert a subtle effect on the toxin to antitoxin ratio in the cell, which in turn regulates toxin function (Yamaguchi et al. 2011). Usually, the transcription of TA operons is regulated by the binding of antitoxin or by a complex formed of both toxin and antitoxin proteins (Brzozowska and Zielenkiewicz 2013). Antitoxins are more labile and more readily degraded under stressful conditions than toxins, which allows toxins to exert their toxic effects (Yamaguchi et al. 2011). The antitoxin molecules are usually binding partners of toxins, either at a gene level or at a protein level, and they regulate the activity of the toxin (Park et al. 2013). PumB from PumAB TA system has been classified as an HTH-type antitoxin since it contains a predicted HTH DNA-binding domain that is characteristic of the Xre family of antitoxins whereas the *pumA* gene encodes a putative RelE-type toxin, suggesting that these genes encode a hybrid TA system (Hernández-Ramírez et al. 2017), where the PumB could play a role in regulating the transcription of the genes from *pumAB* operon.

Our findings indicated that the PumAB system is regulated at the transcriptional level by the PumB antitoxin, which also has the potential to function as an RNase that degrades specifically the mRNA of *pumA*.

Materials and Methods

Bacterial strains, culture conditions, and plasmids

91 *P. aeruginosa* PAO1 (Li et al. 2007) is a standard prototrophic strain employed as a
92 host for recombinant plasmids. *E. coli* BL21-CodonPlus® (DE3)-RP (Stratagene, San
93 Diego, CA, USA) was used for protein overexpression. PAO1-bearing plasmid
94 pUM505 (Hg^R, Cr^R) (Cervantes et al. 1990) was employed to evaluate the
95 transcriptional expression of *pumAB* genes. *P. aeruginosa* PAO1(pUM505)
96 transformed with the pUC_pumB recombinant plasmid was used for the analysis of
97 the effect of PumB in the expression of genes plasmid and chromosomally encoded.

98 The culture media used were Nutrient Broth (NB) and Luria-Bertani broth (LB); for
99 solid media, 1.5% agar was added (Green and Sambrook 2012), and the M9 salts
100 minimal medium (Sigma, St. Louis, MO, USA) was supplemented with 20 mM
101 glucose and 0.1 mM CaCl₂. When necessary, Ampicillin (100 µg ml⁻¹) or Carbenicillin
102 (400 µg ml⁻¹) was added to the agar plates. Cultures were routinely grown overnight
103 at 37°C on a shaker, and growth was monitored as optical density (OD) at 600 nm
104 (OD₆₀₀) with a spectrophotometer.

105 The pUC_pumA, pUC_pumB and pUC_pumAB recombinant plasmids, containing
106 the respective genes *pumA*, *pumB*, or *pumAB*, cloned with their own promoters into
107 the pUCP20 *Escherichia/Pseudomonas* shuttle vector (Hernández-Ramírez et al.
108 2017), were used for gene expression and oxidative stress assays. The
109 pTrcHis_pumA and pTrcHis_pumB plasmids contain the *pumA* and *pumB* genes
110 cloned into the pTrcHisC or pTrcHis2A vectors, respectively. These plasmids were
111 utilized individually for the overexpression of different Pum proteins, and together at
112 pTrcHis_pumAB (that contains both *pumA* and *pumB* genes) for gene expression
113 analysis (Hernández-Ramírez et al. 2017).

114

115 **Genetic techniques and sequence analysis**

116 Molecular genetics procedures were carried out by following standard methods
117 (Green and Sambrook 2012). Probable promoter sequences were searched with the
118 Neural Network Promoter Prediction program
119 (http://www.fruitfly.org/seq_tools/promoter.html).

120

121 **RNA isolation and qRT-PCR assays**

122 For the measurement of *pumA* and *pumB* gene expression, cultures from *P.*
123 *aeruginosa* PAO1 and *E. coli* BL21 with recombinant plasmids were harvested by
124 centrifugation after being grown for different time periods; *E. coli* BL21 cultures were
125 induced with 1 mM of IPTG. Total RNA was isolated and quantified as described
126 previously by (Hernández-Ramírez et al. 2017). For qRT-PCR of the *pumA* gene
127 and the 16S rRNA reference gene, oligonucleotide primers and hydrolysis probes
128 were used as previously described by Hernández-Ramírez (2017). Oligonucleotide
129 primers and hydrolysis probes utilized for the qRT-PCR of the *pumB* gene were
130 designed by employing Biosearch Technologies software
131 (<https://www.biosearchtech.com/support/applications/realtimedesign-software>)
132 purchased from Biosearch Technologies (Novato, CA, USA) (Table S1).
133 Amplification of the *pumA* and *pumB* genes was performed using the 5' exonuclease
134 probe qRT-PCR method. qRT-PCR was performed with total RNA samples (500 ng)
135 and the SuperScript III Platinum One-Step qRT-PCR Reagent kit (Thermo Fisher

Scientific, Waltham, MA, USA) on the LightCycler 480 II System (Roche Molecular
Diagnostics, Pleasanton, CA, USA). Amplification signal curves were analyzed at
absorption wavelengths of 530 nm. Appropriate positive and non-template controls
were included in each test run. Relative expressions of the *pumA* and *pumB* genes
as well as the chromosomal and plasmid encoded genes from PAO1 and pUM505,
respectively, were normalized with expression values obtained from 16S rRNA
genes from *P. aeruginosa* (accession number AE004091; region: 722096–723631)
and *E. coli* (accession number AJ605115). Estimation of relative gene expression
was performed via the classical calibration dilution curve and slope calculation. A
five-fold dilution series (500 ng 0.05 ng total RNA) was prepared and utilized as
sample in the qRT-PCR. Efficiency (E) was obtained from standard curves utilizing
the formula $E = (10^{-1/\text{slope}} - 1) \times 100$. Relative expression levels were determined with
the efficiency correction method, using amplification efficiencies between target and
reference genes (Pfaffl 2001).

For the analysis of the role of PumB as a regulator of the expression of genes
chromosomal and plasmid encoded, oligonucleotide primers and hydrolysis probes
for the PAO4674, PAO729 and PAO125 genes from the chromosome of *P.*
aeruginosa PAO1 strain and the *orfs* 35, 36 and 131 from pUM505 plasmid were
designed (Table S1). For the measurement of expression of chromosomal and
plasmid encoded genes, cultures from *P. aeruginosa* PAO1 (pUM505) strain
transformed with the pUC_pumB recombinant plasmid were harvested by
centrifugation after being grown for different time periods and total RNA was isolated
and quantified as described previously by (Hernández-Ramírez et al. 2017).

Amplification of the chromosomal and plasmid encoded genes was performed using the 5' exonuclease probe qRT-PCR method as was described previously.

Oxidative stress assays

To induce oxidative stress, cultures of *P. aeruginosa* PAO1 (pUC_pumA), *P. aeruginosa* PAO1 (pUC_pumB), and *P. aeruginosa* PAO1 (pUC_pumAB) were grown in NB and incubated separately under two different conditions: 1) in the absence of H₂O₂, and 2) in the presence of 0.02 M H₂O₂. The time at the start of incubation was noted as time zero. Growth was monitored at different intervals by measuring OD₆₀₀. *P. aeruginosa* PAO1 (pUCP20) was employed as negative control. Additionally, samples of 2 ml were taken from the culture for RNA isolation.

Overexpression and purification of toxin–antitoxin proteins

Overnight cultures of *E. coli* BL21 (pTrcHis_pumA), *E. coli* BL21 (pTrcHis_pumB), and *E. coli* BL21 (pTrcHis_pumAB) were diluted to 1:50 in 250 ml of fresh NB and incubated on a shaker at 37°C, until the value of OD₆₀₀ was 0.5. Overexpression of His-PumA and PumB-His individual proteins was induced with 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG), and the cultures were incubated for an additional 6 h. Overexpression of PumA/PumB-His (in co-expression) was induced with 1 mM IPTG and the cultures were incubated additionally for 15 min, 1 h, 3 h, and 24 h. Overexpression of Pum proteins was observed by SDS-PAGE using a Tris-glycine buffer as described by Hernández-Ramírez et al. (2017).

Purification of His-PumA and PumB-His proteins was performed as described by Hernández-Ramírez et al. (2017).

Electrophoretic Mobility Shift Assays (EMSA)

DNA fragments containing the putative promoter regions of the *pumAB* operon and the *pumB* gene were amplified from *P. aeruginosa* (pUM505) DNA by PCR, using specific oligonucleotides (Table S1). Purified DNA fragments (200 ng) were incubated with purified PumB-His protein and/or His-PumA protein in 20 μ L of binding buffer [10 mM Tris/HCl (pH 8.0), 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 5% glycerol, 0.5 mM DTT, and 0.1 mg BSA] and incubated for 1 h at 37°C. Binding reaction mixtures were loaded on 6% native PAGE gels, separated at 100 V for 45 min in TAE buffer (Green and Sambrook 2012), and stained with 0.01% ethidium bromide for 30 s.

Palindromic region deletion of *pumAB* operon by fusion PCR

The palindromic region of *pumAB* operon of pUM505 was deleted by fusion PCR. For palindromic deletion, we designed a pair of oligonucleotides without the respective nucleotides of the palindromic region (Table S1). For the deletion, three PCR fragments were amplified: (1) a PCR fragment was obtained using the oligonucleotide ODirpAB (forward) and the specific oligonucleotide ORev $\square$ pAB (reverse) for deletion, (2) the second PCR fragment was obtained using a specific oligonucleotide ODir $\square$ pAB (direct) for deletion and the oligonucleotide ORevpAB (reverse), and (3) a PCR fragment, containing the promoter/operator PpumAB

promoter without the palindromic region, was obtained using the oligonucleotides ODirpAB and ORevAB, and the fragments 1 and 2 as template. The PCR fragment of 275 pb containing the deletion of the palindromic region was employed to EMSA assays as was described previously.

mRNA stability assays

Total RNA (400 ng) obtained from cultures of *E. coli* BL21 (pTrcHis_pumA), *P. aeruginosa* (pUC_pumB), and *E. coli* BL21 (pTrcHis_pumAB) was incubated separately under two conditions: 1) in the absence of PumB-His, and 2) in the presence of 0.1 to 1.0 μ g of PumB-His purified protein for different intervals of time at 37°C. After incubation, the total RNA was employed to determine the mRNA of *pumA* or *pumB* by qRT-PCR, using the oligonucleotides and probe previously described (Hernández-Ramírez et al. 2017). The same strategy was employed in using His-PumA protein and CrpP-His protein (Chavéz-Jacobo et al. 2018) as negative controls. To determine ribonuclease (RNase) activity, 400 ng of total RNA was incubated with 1.0 μ g of PumB-His purified protein for 1 h at 37°C. The incubation was performed under two different conditions: in the absence of, and in the presence of ribonuclease inhibitors RNasin (Promega) or RNaseOUT (Invitrogen). Following this, the mRNA of *pumA* and *pumB* were quantified by qRT-PCR as previously described.

Statistical analysis

Data analysis was performed with GraphPad Prism 5 software. Analysis of Variance (ANOVA) employing the Tukey test or Student *t* test was performed to analyze the expression assays. *P* < 0.05 was considered statistically significant.

Results

The *pumAB* operon possess multiple putative promoters

The PumAB TA system is encoded by the *pumAB* genes that are organized into an operon, and co-transcribed into a bicistronic mRNA (Hernández-Ramírez et al. 2017). In a previous analysis of the DNA sequence of the *pumAB* operon, a putative promoter σ^{70} type located at the 5' end at the *pumA* gene was identified (Hernández-Ramírez et al. 2017); this was denominated P_{pumAB} . However, a new analysis showed that two additional putative promoters were located at the 5' end at the *pumB* gene (Fig. 1). One of the putative promoters was similar to σ^{70} -type promoter and was denominated P_{pumB} , while the other promoter was similar to the AlgU (σ^E)-type promoters (Fig. 1a) and was denominated $P_{pumB-AlgU}$. In *P. aeruginosa* AlgU is a σ factor implicated in the transcription of genes involved in resistance to oxidative stress and heat-shock (Potvin et al. 2008). This analysis suggested a differential regulation of *pumAB* genes by multiple promoters.

Differential expression of the *pumAB* genes

It was described that *pumA* and *pumB* genes are co-transcribed as bicistronic mRNA (Hernández-Ramírez et al. 2017), suggesting that they form an operon. We determined the levels of expression of the *pumA* and *pumB* genes by qRT-PCR

assays using DNA from *P. aeruginosa* (pUM505), where the *pumAB* operon is expressed by the P_{pumAB} putative promoter. The results showed that the amount of *pumB* mRNA was around 100 times more than that of *pumA* mRNA (Fig. 2a), suggesting a differential regulation of the *pumAB* genes, probably through the expression of *pumA* and *pumB* genes by the P_{pumAB} promoter and the individual expression of *pumB* by its own promoters. To analyze mRNA levels without the regulation of the P_{pumAB} promoter, RNA isolated from *E. coli* BL21 (pTrcHis_*pumAB*), in which the *pumAB* operon was transcribed by the Ptr promoter (induced by the IPTG addition), was employed. The results were similar to those obtained when the *pumAB* operon contained the P_{pumAB} promoter (Fig. 2b). The *pumA* and *pumB* mRNA levels from cultures grown for 15 min did not exhibit statistical differences between them. However, after incubation of 1, 3, and 24 h, the levels of *pumB* mRNA were around 3, 5, and 5 times higher than those of *pumA* mRNA, respectively (Fig. 2b). These results suggested that when the incubation period is short, both the genes are co-transcribed, whereas for longer incubation periods, the mRNA levels of both genes are different, probably due to a particular regulation in *pumB* gene expression. However, another possibility for the differences between the *pumA* and *pumB* mRNA levels after 1 h of incubation could be the degradation of *pumA* mRNA.

We also analyzed whether the expression of *pumA* and *pumB* genes is regulated differentially by changes in growth conditions and examined the effect of growth in minimal medium M9. Growth kinetics showed that in the medium M9, the growth of *P. aeruginosa* PAO1 (pUC_*pumAB*) and *P. aeruginosa* PAO1 (pUCP20) was similar (Fig. S1, Supplementary Material). On using this medium of growth, the mRNA levels

of *pumB* were found to be higher (around 6, 7, and 8 times more at 4, 8, and 12 h, respectively) in comparison with *pumA* from *P. aeruginosa* PAO1 (pUC_*pumAB*) (Fig. 2c). This behavior was similar to that observed when the bacterium was grown in nutritive medium (Fig. 2a and b). Collectively, these results indicated that *pumAB* operon genes are regulated differentially.

***pumB* is expressed by its own promoters**

To analyze whether *pumB* could be transcribed without *pumA*, by its own promoters (P_{pumB} and/or $P_{pumB-AlgU}$), qRT-PCR assays were performed using total RNA isolated from *P. aeruginosa* PAO1 (pUC_*pumB*), which possesses the *pumB* gene cloned alone. Cultures for RNA isolation were obtained from the exponential growth phase (4 h) and from the stationary growth phase (8 h). The data revealed that *pumB* could be transcribed individually by its own promoters (Fig. 3). The mRNA level of *pumB* was around three times higher in the exponential phase than in the stationary phase, suggesting that cellular conditions associated with the growth phase could modulate the *pumB* expression.

Oxidative stress modulates the expression of the *pumB* gene

To analyze whether the expression of *pumA* and *pumB* genes is regulated differentially by oxidative stress, we examined the effect of H₂O₂ on *pumA* and *pumB* expression during the bacterial growth. Growth kinetics showed that *P. aeruginosa* PAO1 (pUC_*pumAB*) was more susceptible to oxidative stress than *P. aeruginosa*

PAO1 (pUCP20) (Fig. 4a), suggesting that oxidative stress regulates the PumAB TA system at the level of protein stability or at the transcriptional level. In an attempt to explain this, *pumAB* expression was analyzed under conditions of oxidative stress by qRT-PCR at 4, 8, and 12 h. When *P. aeruginosa* PAO1 (pUC_*pumAB*) was grown under oxidative stress, the mRNA levels of *pumB* were four times greater than *pumA* levels, in samples obtained from the exponential phase (4 h) (Fig. 4b). Interestingly, this behavior shifted in samples obtained from the stationary phase where the mRNA levels of *pumB* decreased 1.5 and 4 times of *pumA* levels for samples from 8 and 12 h, respectively (Fig. 4b). These results indicate that the expression of the *pumB* gene could be regulated by stress conditions. To analyze the effect of oxidative stress on the individual expression of *pumA* and *pumB* genes, total RNA from cultures of *P. aeruginosa* PAO1 (pUC_*pumA*) and *P. aeruginosa* PAO1 (pUC_*pumB*), grown with or without H₂O₂ were employed. In *P. aeruginosa* PAO1 (pUC_*pumA*), the *pumA* expression was derived from the P_{*pumAB*} promoter, whereas in *P. aeruginosa* PAO1 (pUC_*pumB*), the *pumB* expression was derived from the P_{*pumB*} and/or P_{*pumB-AlgU*} promoters. Our results showed that the transcripts of both genes were decreased by three times during the stationary growth phase, with respect to the levels observed in the exponential phase (Figs. 4c and 4d). Interestingly, the mRNA of *pumB* decreased when the strain was grown in the presence of H₂O₂, both in the exponential phase (decreased by 6 times) and the stationary phase (decreased by 11 times) (Fig. 4c), indicating that oxidative stress decreased the individual expression of *pumB*. The mRNA levels of *pumA* did not change when the cultures were grown in presence of H₂O₂ (Fig. 4d), suggesting that oxidative stress conditions do not regulate the P_{*pumAB*} promoter that controls the

pumA and *pumB* co-expression. Collectively, these results indicated that *pumAB* operon genes are regulated differentially, and *pumB* expression is regulated by oxidative stress in individually.

PumB Binds Directly to the 5' Region of the *pumAB* Operon

It has been reported that the PumB protein possesses an HTH DNA binding domain (Hernández-Ramírez et al. 2017) (Fig. 1b), suggesting that it could play a role in regulating the transcription of the genes from *pumAB* operon. To determine whether PumB can bind to the promoter/operator region of the *pumAB* operon, we conducted an electrophoretic mobility shift assay (EMSA). The 5' end region of the *pumAB* operon, where the P_{pumAB} promoter is located, was amplified by PCR (Fig. 5), and the amplified product (denominated PpumAB) was incubated with the purified PumB protein. The results showed that the PumB protein could retard the mobility of PpumAB DNA in a dose-dependent manner (Fig. 5b, lines 1-5), indicating that the PumB protein is bound to the 5' promoter/operator region of the *pumAB* operon, forming a PpumAB DNA-PumB complex. These results suggested that PumB regulates the expression of the genes in the operon that encode the PumAB toxin-antitoxin system.

Previously, the P_{pumB} and $P_{pumB-AlgU}$ promoters were identified at the 5' end region of the *pumB* gene in the *pumAB* operon. To determine whether PumB regulates its own expression, we analyzed the ability of PumB to bind to the region of DNA where the P_{pumB} and $P_{pumB-AlgU}$ promoters are located. For this analysis three PCR fragments

were used: 1) $\square$ PpumAB, a PCR fragment of 0.42 Kb that in addition to containing the coding region of *pumA* gene, possesses 43 nucleotides (nt) upstream to the start codon of this gene, where the putative RBS site is located, but without the P_{pumAB} promoter; 2) PpumB/PpumB-AlgU, a PCR fragment of 0.29 Kb where the P_{pumB} and $P_{pumB-AlgU}$ promoters are located; and 3) $\square$ ppumB/ppumB-AlgU, a PCR fragment of 0.45 Kb that contains the coding region of *pumB* gene and the region where the putative RBS site for the *pumB* transcript is located, but without the P_{pumB} and $P_{pumB-AlgU}$ promoters. The EMSA showed that PumB was bound to $\square$ PpumAB DNA, suggesting that PumB can bind to a promoter/operator region where P_{pumB} and $P_{pumB-AlgU}$ promoters are located (Fig. 5c). However, when the PpumB/PpumB-AlgU DNA fragment was used, PumB was not able to bind (Fig. 5c). Additionally, PumB did not bind at the *pumB* coding region of the $\square$ ppumB/ppumB-AlgU DNA lacking the P_{pumB} and $P_{pumB-AlgU}$ promoters region (Fig. 5c). These assays suggested that PumB does not regulate the individual expression of *pumB* gene, and that the PumB binding region (operator) is located downstream of the P_{pumAB} promoter.

By *in silico* analysis of the $\square$ PpumAB DNA region, we identified one palindromic region, GTGATTTATAAGTCAC, (underline Indicates the palindromic region) adjacent to the putative RBS site of the *pumA* gene (Fig. 1a), suggesting that these nucleotides could comprise the binding site of PumB. To determine the ability of PumB to bind to this region of DNA we employed a PCR fragment of 0.189 kb denominated as pumAB', located between the nt 43 upstream and the nt 146 downstream to the start codon of the *pumA* coding region where the palindromic region is located (Fig. 5a). EMSA assays showed that PumB retards the mobility of

the PpumAB' region in a dose-dependent manner (Fig. 5d), suggesting that PumB can bind to the palindromic region. In addition, with the aim to demonstrate if the palindromic region is necessary for the PumB binding a DNA fragment of 275 pb of the *pumAB* operator/promoter region with a deletion of the palindromic region was generated by fusion PCR (denominated $\square$ PpumAB') and EMSA assays were conducted. The results showed that PumB is not able to binding to the $\square$ PpumAB' region (Fig. 5e), confirmand that PumB is binding to the palindromic region to regulate the *pumAB* operon expression.

Additionally, to demonstrate that PumB is only able to bind to promoter/operator specific regions, the PumB protein was incubated with the PchrS PCR fragment. In this fragment, DNA is located in the P_{chrs} promoter/operator region, which is recognized by the chromate resistance regulator (ChrS), as previously reported by Aguilar-Barajas et al. (2015). The EMSA showed that the PumB protein does not bind to the Pchrs DNA (Fig. 5b, lines 6-9), indicating that PumB recognizes only a specific operator/promoter region. Taken together, these results suggest that PumB regulates the *pumAB* gene co-expression through the binding at the P_{pumAB} promoter/operator region, and that *pumB* gene could also be expressed individually through its own promoters without PumB regulation.

PumA improves the binding of PumB to DNA

With the aim of evaluating whether the binding of PumB to DNA could be modulated by the presence of its cognate toxin, we performed EMSA assays with and without

the addition of the PumA protein. The PpumAB DNA region, where the P_{pumAB} promoter is located, was incubated with mix PumB-His and His-PumA proteins. The results showed that PumA was not able to bind to the DNA of P_{pumAB} without PumB (Fig. 6). However, a greater retardation of the PpumAB DNA fragment was observed when the DNA was previously incubated with both PumB and PumA, in comparison with DNA incubated only with PumB; an effect that was dose-dependent on the PumA concentration (Fig. 6). This observation suggests the formation of a PpumAB DNA-PumA-PumB complex. In addition, to determine whether PumA can bind to the palindromic region adjacent to the putative RBS site of the *pumAB* operon, we analyzed the ability of PumA to bind to the PpumAB' region. The results showed that PumA was not able to bind to the DNA of PpumAB' without PumB (Fig. S2). Moreover, these results suggest that the PumA protein is joined at the DNA through its interaction with the PumB protein, which binds to the DNA operator region of the *pumAB* operon. Since both PumA and PumB are recombinant proteins that possess a His-tag, it was possible that the His-tag was responsible for binding to DNA. To test this, we carried out an EMSA assay using the PpumAB DNA fragment and the CrpP-His recombinant protein, a protein involved in resistance to Ciprofloxacin (Chávez-Jacobo et al. 2018). The assay demonstrated that CrpP-His could not bind to this DNA (Fig. S3, Supplementary Material), indicating that the PumB and PumA binding is not facilitated by the His-tag of the proteins.

PumB regulate the expression of genes

405 As was described the PumB protein possesses an HTH DNA binding domain
406 (Hernández-Ramírez et al. 2017) (Fig. 1b), and could binding to the
407 operator/promoter region located downstream of the P_{pumAB} promoter (Fig. 5),
408 suggesting that PumB regulate the transcription of the *pumAB* genes. With the aim
409 to determine if PumB could be a general regulator of the expression of genes, we
410 analyzed the effect of the expression of *pumB* in the levels of expression of PAO125,
411 PAO729 and PAO4674 (chromosomic-encoded genes) from *P. aeruginosa* PAO1
412 and the *orf35*, *orf36* and *orf131* (plasmid-encoded genes) from pUM505. PAO125,
413 PAO729 and PAO4674 encodes to an antitoxin type RelB, a toxin type ParE and an
414 antitoxin type HigA homologue proteins, respectively, whereas the *orf35*, *orf36* and
415 *orf131* encode to two hypothetic proteins and the CrpP enzyme (that confers
416 ciprofloxacin resistance), respectively. To conduct this the *P. aeruginosa* PAO1
417 (pUM505) strain was transformed with the pUC_pumB recombinant plasmid and the
418 mRNA levels of the genes were determined. The results showed that the
419 transference of *pumB* gene regulates positive or negatively the expression of genes;
420 under the expression of *pumB* the levels of expression of the PAO4674 and PAO729
421 genes from cultures obtained from both exponential and stationary phases of growth
422 were increased (Fig. 7a and b), whereas the mRNA levels of PAO125 gene and
423 *orf35* were decrease in both phases of growth (Fig. 7c and d). The mRNA of *orf36*
424 only was modified in cultures from the stationary phase of growth and the expression
425 of the *orf131* was not modified under the *pumB* expression (Fig. 7e and f). These
426 data suggest that PumB additionally to regulate the *pumAB* operon could be a
427 general regulator for the expression of genes.

PumB degrades *pumA* mRNA

To analyze whether PumB has the potential to degrade *pumA* mRNA, the stability of the *pumA* transcript was determined after its incubation with the antitoxin. Total RNA isolated from *E. coli* BL21 (pTrcHis_*pumA*) was incubated in the presence and absence of the PumB purified protein, and the mRNA level of *pumA* in both the cases was determined by qRT-PCR. The results showed that the mRNA level of *pumA* decreased by 55% after its incubation with PumB, as compared to incubation without PumB (Fig. 8a). A correlation was observed between the decrease in the mRNA levels of *pumA* (with respect to PumB) and incubation time. It was found that more than 65% of mRNA diminishes after 60 min of incubation (Fig. 8b), suggesting that the mRNA of *pumA* is degraded by the PumB protein. To determine whether the PumB activity was specific to the *pumA* transcript, we determined the rRNA levels of the 16S RNA gene after its incubation with PumB. The results showed that PumB did not affect the 16S rRNA levels (Fig. S4a and b, Supplementary Material). With the aim of analyzing the effect of PumB on the stability of another mRNA, we used the total RNA from the *P. aeruginosa* PAO1 and determined the mRNA levels of PA_729 gene. The PA_729 gene is predicted to encode a protein homolog of ParE toxin of TA systems that is located in the chromosome of *P. aeruginosa* PAO1 without an antitoxin gene adjacent, suggesting that the PA_729 gene encodes a putative orphan toxin module. The results showed that PumB does not affect the mRNA levels of PA_729 (Fig. S4c, Supplementary material). Therefore, these data suggest that PumB does not affect the stability of mRNAs other than *pumA*. With the aim of establishing whether PumB also degrades its own transcript, total RNA from

451 *P. aeruginosa* PAO1 (pUC_pumB) was incubated with and without PumB protein.
452 The results showed the same mRNA levels of *pumB* in the presence and absence
453 of the protein, suggesting that PumB does not degrade its own transcript (Fig. 8c).
454 In addition, as it has been reported that several toxins such as the proteins RelE,
455 YoeB, and YafO from *E. coli* (Masuda and Inouye 2017) and VapC from
456 *Mycobacterium tuberculosis* (Mckenzie et al. 2012) possess RNase activity, we
457 analyzed whether PumA could degrade its own transcript, as well as other mRNA.
458 The results showed that PumA does not modify the mRNA levels of its own
459 transcript, neither does it degrade *pumB* or 16S rRNA (Figs. 8d and S4), suggesting
460 that it has some other function.

461 Since *pumA* and *pumB* are co-transcribed as bicistronic mRNA, we finally analyzed
462 the effect of PumB on the stability of *pumA* and *pumB* co-transcripts. For this, total
463 RNA from *E. coli* BL21 (pTrcHis_pumAB) was incubated in the presence or absence
464 of PumB during different time of incubation and the mRNA levels of each *pum* gene
465 were determined. Interestingly, the results showed that *pumA* mRNA levels do not
466 decrease after its incubation during 30 min with the PumB protein; however, the
467 mRNA levels of *pumA* decrease after 1 hour of incubation in presence of PumB and
468 this decrease was higher after 2 hours (Fig. 8e), whereas that *pumB* mRNA levels
469 do not change due to the addition of PumB protein at the times assayed (Fig. 8f),
470 indicating that PumB regulate the expression of *pumAB* by ribonuclease activity on
471 *pumA* mRNA. Together, these results indicate that PumB has the potential to
472 function as RNase which specifically degrades the *pumA* mRNA and avoids its
473 translation.

To demonstrate the RNase activity of the PumB protein, we conducted assays of stability of *pumA* mRNA in the presence of the PumB protein with the addition of RNases inhibitors. The results showed that, in the presence of RNaseOUT, the mRNA levels of *pumA* increased by at least two times with respect to the mRNA levels of *pumA* when it was incubated with PumB protein (Fig. 8g). Similar results were obtained when another RNase inhibitor (RNasin) was added to the mixture *pumA* mRNA and PumB protein. Under this condition, the mRNA levels of *pumA* increased two-fold with respect to when the mRNA of *pumA* was incubated with PumB and without RNasin addition (Fig. 5S). These results indicated that the RNase inhibitors were able to inhibit PumB activity on the mRNA of *pumA*, suggesting that PumB possesses an RNase activity on the *pumA* transcript.

Discussion

Genes that encode TA systems are widely distributed in the plasmids and chromosomes of bacteria, and they protect the bacterial populations under conditions of stress (Gerdes et al. 2005; Harms et al. 2016). The expression of genes encoding TA systems must be in balance, so that the controlled production of the toxin and the antitoxin is achieved by the transcriptional regulation of TA operons (Boss et al. 2013). Generally, in type-II TA systems, the complex formed between the toxin and the antitoxin represses the transcription of TA operons, because the antitoxins are capable of binding DNA via their DNA-binding domain (Goeders and Van Melder 2014). Transcriptional auto-regulation has been described for many type-II TA systems, usually through the mechanism of conditional cooperativity,

which defines the state of transcription using the molar ratio of the toxin to the antitoxin within a cell (Page and Peti 2016).

The PumAB TA system was identified in the pUM505 plasmid from *P. aeruginosa* (Hernández-Ramírez et al. 2017). This system is formed by the toxin PumA and the antitoxin PumB. Like other TA systems encoded in plasmids, the PumAB system also confers plasmid stability, whereas the expression of *pumA* increased the virulence of *P. aeruginosa* and *E. coli*, an effect that is neutralized by the *pumB* expression (Hernández-Ramírez et al. 2017). The PumAB system was found to be a non-conventional TA system, because the genes of the *pumAB* operon are organized differently from the majority of II-type TA systems, and the proteins of PumAB system are not canonical; PumA is a toxin RelE-type protein, whereas PumB is similar to antitoxins HTH-type (Hernández-Ramírez et al. 2017) (Fig. 1b). Based on the characteristics of the PumAB system with respect to its organization and its role in bacterial virulence, we studied the mechanism of regulation of this TA system.

To determine the mechanism of regulation of the PumAB system, we first performed an analysis of the putative regulatory elements located in cis of the *pumAB* operon. In addition to a putative σ^{70} type promoter, located upstream of the *pumAB* operon, identified previously by Hernández-Ramírez et al. (2017), this operon possesses two additional putative promoters, the P_{pumB} promoter (σ^{70} -type) and the $P_{pumB-AlgU}$ promoter (AlgU (σ^E)-type), located upstream of the *pumB* gene, suggesting a complex regulation of the genes. The presence of an internal promoter in TA operons of type-II systems was previously reported. In *Enterococcus faecium*, the *axe-txe* operon possesses a major promoter upstream of the operon and an internal

promoter that regulates the *axe* gene expression (Boss et al. 2013). More recently, from a study of 10 type-II TA systems from *E. coli* K12 str. MG1655, it was determined that the *rnIAB* operon possesses two promoters and produces two transcripts: a transcript of the whole operon (both toxin and antitoxin mRNA), and a transcript that can only be translated into the antitoxin (Deter et al. 2017).

Analysis of the expression of the *pumAB* operon revealed that in the *P. aeruginosa* (pUM505) strain, the *pumA* and *pumB* genes are transcribed differentially, with *pumB* mRNA being more abundant than the *pumA* transcript. Interestingly, the same differences in the mRNA levels of both genes were observed when the assays were conducted with cultures of *E. coli* BL21 (pTrcHis_*pumAB*) where the *pumAB* operon expression was under the control of an induced promoter. This result was not expected since that the *pumA* and *pumB* genes were supposed to be co-transcribed as a bicistronic mRNA (Hernández-Ramírez et al. 2017). Similar results were reported for the *rnIAB* operon of *E. coli* K12 str. MG1655, which showed a different antitoxin-to-toxin RNA ratio (Deter et al. 2017). However, the results shown by Deter et al. (2017) showed that most of TA systems produced a single transcript, in which the mRNA concentration of antitoxin was approximately equal to the concentration of the toxin, suggesting that these systems are apparently not differentially regulated at the transcriptional level and that different translational rates of the two proteins are responsible for the higher production of the antitoxin protein than of the toxin protein (Deter et al. 2017). The *pumB* / *pumA* ratio could suggest that the overproduction of *pumB* is import to the bacteria physiology since it has been

described that the antitoxins are unstable at difference to the toxins and therefore must be generated at a higher rate to keep toxins inactive.

Moreover, it was reported that the medium in which bacterial strains are grown alters the expression of important genes, mentioning that the growth of *E. coli* in minimum medium increased expression of genes of amino acid biosynthetic pathways (Tao et al., 1999), whereas in *Salmonella enterica* serovar Typhimurium, growth in MOPS (minimal medium) and MEM (minimum essential medium) resulted in an increase in the expression of genes of virulence from SPI-2 (*Salmonella* Pathogenicity Island 2), as compared to the levels after growth in LB (Blair et al., 2013). However, the analysis of expression of *pumA* and *pumB* genes from *P. aeruginosa* (pUC_pumAB) grown in minimal medium M9 showed higher levels of *pumB* mRNA than that of *pumA* mRNA, showing the same pattern of expression of both the genes with respect to that observed when nutrient rich medium was used, suggesting that the expression of *pumA* respect to the *pumB* levels is not altered by the medium of growth used. Additionally, the analysis of the expression of *pum* genes from *E. coli* BL21 (pTrcHis_pumAB) strain showed that at early exponential growth phase, no difference was observed between the two transcripts, suggesting that a differential regulation of the genes is determined by the growth phase. In type-II TA systems, the ratio of antitoxin to toxin is higher in the susceptible population and smaller during persistence (a small fraction of bacteria that demonstrate resistance to antibiotics without genetic change). In the persistent state, the cellular translation rate is low and the low antitoxin / ratio derepresses the transcription of TA loci generating a high rate of TA transcription and toxin production (Gerdes and Maisonneuve 2012). The

565 differences in ratio of transcription of *pumA* /*pumB* favoring the level of transcript of
566 *pumB* during stationary phase of growth could suggest that the overproduction of the
567 antitoxin, under this condition of growth, is important to the control of the toxin
568 effects. However, another explanation for the difference in the mRNA levels of *pum*
569 genes could be related to the individual expression of *pumB* by the P_{pumB} and P_{pumB-}
570 *AlgU* promoters. Additionally, it was observed that *pumB* could be expressed
571 individually showed a greater transcript during the exponential phase as compared
572 to the stationary phase. This strengthens the hypothesis that the expression of *pum*
573 genes can be regulated differentially by external and internal promoters of the
574 operon and by the stage of cell growth.

575 It has been reported that under normal growth conditions, TA systems remain
576 inactive and they can be temporarily activated in response to environmental stress,
577 as well as nutrition and oxidative stress (reviewed by Hůrak and Tamman 2017). The
578 pattern of expression of *pum* genes under stressful conditions was similar to that of
579 the non-stressful conditions during the exponential growth phase; however,
580 expression levels of *pumB* mRNA with respect to *pumA* changed during the
581 stationary growth phase, showing 50% less of the *pumB* transcript. In addition, the
582 oxidative stress only affected the individual expression of the *pumB* gene and not
583 the *pumA* gene. The negative regulation of the *pumB* gene under oxidative stress
584 was not expected, and this suggested that the $P_{pumB-AlgU}$ promoter controls the
585 expression of *pumB* in response to oxidative stress during the stationary growth
586 phase.

587 The PumB protein possesses an HTH DNA binding domain (Hernández-Ramírez et
588 al. 2017), suggesting that it could act as a transcriptional regulator of the genes in
589 the *pumAB* operon. DNA binding in antitoxins mediates the transcriptional auto-
590 regulation of their own operons; therefore, the expression and activation of TA
591 systems are tightly regulated by their cognate antitoxins at both transcriptional and
592 post-transcriptional levels (reviewed in Schuster and Bertram 2013). By EMSA
593 assays we observed that the PumB protein could bind to the 5' end of DNA region
594 where the promoter of *pumAB* operon was located, forming a P_{pumAB} DNA-PumB
595 complex, suggesting that the PumB antitoxin functions as a transcriptional regulator
596 of the *pumAB* operon. The putative region where the operator was located was
597 situated into a palindromic sequence located between the P_{pumAB} promoter and the
598 star codon of the *pumA* coding region, since the deletion of this palindromic
599 sequence prevents the recognition of PumB. In type-II TA systems the transcriptional
600 auto-regulation of the TA operons is generally promoted by the antitoxins or
601 antitoxin–toxin complexes that repress transcription through their binding to the
602 palindromic sequences in their promoters (reviewed by Otsuka 2016). PumB was
603 unable to bind to the P_{pumB} and $P_{pumB-AlgU}$ promoters/operator region, located at the
604 5' end region of the *pumB* gene, suggesting that PumB does not regulate its own
605 individual expression. The ability of PumB to bind specifically at the
606 promoter/operator region of the *pumAB* operon, and not to P_{pumB} and $P_{pumB-AlgU}$
607 promoters regions suggested that the *pumAB* operon regulation is complex, where
608 the transcription of the bicistronic mRNA (that possesses both toxin and antitoxin
609 mRNA) transcription is regulated by the PumB antitoxin, and that the individual
610 expression of the *pumB* gene is regulated by the AlgU factor.

The participation of the PumA toxin in the PumB mediated regulation of the *pumAB* operon was analyzed, and the results showed that under the conditions assayed, PumA was not able to bind to the DNA of the P_{pumAB} promoter/operator region of the *pumAB* operon without PumB. However, the binding of PumB was improved by PumA in a dose-dependent manner, depending on the concentration of PumA, suggesting the co-regulation of *pumAB* operon expression by the PumB-PumA complex. Usually, type-II antitoxins auto-repress their own promoter, both alone and in complexes with their cognate toxins, and the transcriptional regulation of some TA systems is dictated by the stoichiometry between toxin and antitoxin proteins, which is termed as conditional cooperativity (Schuster and Bertram 2013). Moreover, in type-II TA systems, the simple repression by promoter binding could be enhanced by conditional cooperativity (Rocker and Meinhart 2016). In addition, we determine that the PumB protein could be a general regulator of genes at modify the mRNA levels of some genes chromosomic or plasmid encoded, shown a similar role than other antitoxins previously reported like the antitoxin DinJ a global regulator that represses its locus as well as controls levels of the stationary sigma factor RpoS (Zhao et al. 2016).

As previously stated, *pumA* and *pumB* genes are co-transcribed (Hernández-Ramírez et al. 2017); nevertheless, the mRNA levels of both genes are differential. In order to show that the differences in transcript levels between both genes is not due to the degradation of *pumA* mRNA, the effect of PumB on the stability of the *pumA* transcript was determined. Unexpectedly, the addition of PumB specifically decreased the *pumA* mRNA levels (when expressed individually and co-expressed

with *pumB*) and not the other mRNAs assayed, indicating that the antitoxin negatively affects the stability of *pumA* mRNA. The negative effect of PumB on *pumA* transcript stability was inhibited by the addition of RNase inhibitors, suggesting that PumB possesses RNase activity. In general, transcriptional auto-regulation by the toxin and antitoxin has been described for many type-II TA systems (Page and Peti 2016), while in type-V TA systems, the antitoxin protein cleaves the toxin mRNA (Wang et al., 2012). The GhoT/GhoS was the first example of type-V TA systems (Wang et al. 2012). The antitoxin GhoS adopts a ferredoxin-like fold, similar to CRISPR-associated-2 (CAS2) sequence-specific endoribonucleases, and cleaves ghoT mRNA, preventing its translation into the toxin GhoT (Wang et al. 2012). Our results suggested that PumB possesses RNase activity similar to the GhoS antitoxin and has the potential to cleave *pumA* mRNA. However, in contrast with GhoS, PumB shows the ability to bind DNA, suggesting that it regulates the TA operon transcription and is able to

regulate the expression of other genes. And the PumB binding is improved by the PumA protein, suggesting a mechanism of conditional cooperativity.

Our results indicate that the antitoxin PumB regulates the PumAB TA system by functioning as a transcriptional regulator of *pumAB* operon expression. It can also function as RNase, cleaving the mRNA of the toxin, and thereby exhibiting a complex mechanism for the regulation of the PumAB TA system. Taken together, we conclude that the PumAB TA system possesses multiple mechanisms of regulation providing new insights into the understanding of the control of TA systems encoded by mobile plasmid-encoded genes, and being a TA system that could provide to

adaptive properties, such as virulence, to *P. aeruginosa* and possibly to other pathogenic bacteria and may be involved in evolution and pathogenesis.

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Transparency declarations

None to declare.

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Figure Legends.

Figure 1. Analysis of the regulatory region of *pumAB* operon and secondary structure prediction of PumA and PumB toxin-antitoxin proteins. (a) The nucleotides of the coding region for the *pumA* and *pumB* (in italics) genes of the *pumAB* operon are indicated in capital letters. The putative RBS binding sites are indicated in lower-case bold letters, and the start and stop codons are highlighted in bold. The -10 and -35 regions of putative σ^{70} and AlgU promoters are indicated in bold and are underlined. Nucleotides conserved in promoters from *pumA* and *pumB* genes with respect to the sequence consensus of the σ^{70} and AlgU promoters are shown. The palindromic sequences probably involved in PumB binding are indicated with an arrow. **(b)** On top the amino acids sequence of the PumA and PumB from pUM505 are shown and down the secondary-structure elements predicted (using the MINNOU program) are indicated. Barrels correspond to alpha helices and arrows to beta sheets. HTH and RelE/ParE domains for PumA and PumB, respectively, are indicated by rectangles.

Figure 2. Differential expression of *pumA* and *pumB* genes. Total RNA (500 ng) from cultures were used to determine the expression levels of the genes from the *pumAB* operon by qRT-PCR, as described in the Methods section. Expression of *pumA* (white) and *pumB* (grey) genes from **(a)** *P. aeruginosa* (pUM505 cultures grown at the exponential phase; **(b)** *E. coli* BL21 (pTrcHis_*pumAB*) cultures grown for several incubation periods after induction with 1 mM IPTG or **(c)** *P. aeruginosa* PAO1 (pUC_*pumAB*) grown for 4 h, 8 h, and 12 h in M9 medium. Values were

referenced with respect to the transcription of the 16S genes from *P. aeruginosa* (a and c) or *E. coli* (b). Values represent the means of three independent determinations in duplicate, with standard error (SE) bars shown. One-way Analysis of Variance (ANOVA) with Tukey post-hoc test was used to compare the data. The different letters above the bars indicate statistical significance, values with identical letter were not considered significantly different ($p < 0.05$).

Figure 3. *pumB* gene is expressed individually by its own promoters.

Expression of the *pumB* gene from cultures of *P. aeruginosa* (pUC_ *pumB*) at exponential phase (4 h) or stationary phase (8 h). Values represent the means of three independent determinations in duplicate with standard error (SE) bars shown. Values were normalized with respect to the transcription of the 16S gene from *P. aeruginosa*. Student *t* test was used to compare the bars, and significant differences ($p < 0.05$) are indicated with the letters a and b.

Figure 4. Expression of *pumA* and *pumB* genes from the PumAB TA system

are affected under conditions of stress. (a) Cultures of *P. aeruginosa* PAO1 (pUCP20) (○) and *P. aeruginosa* PAO1 (pUC_ *pumAB*) (●) were grown at 37°C with shaking in NB. 0.02 M of H₂O₂ was added to induce oxidative stress, and growth was monitored at different intervals as optical density (OD) at 600 nm (OD₆₀₀) with a spectrophotometer. **(b)** Expression of *pumA* (white) and *pumB* (grey) genes from cultures of *P. aeruginosa* PAO1 (pUC_ *pumAB*) incubated for 4 h, 8 h, and 12 h under

oxidative stress. Individual expression of **(c)** *pumB* and **(d)** *pumA* genes from cultures of *P. aeruginosa* PAO1 (pUC_*pumA*) and *P. aeruginosa* PAO1 (pUC_*pumB*), respectively, incubated for 4 h or 8 h with (bars with lines) or without (full bars) inducing oxidative stress. Values represent the means of three independent determinations in duplicate with standard error (SE) bars are shown. Values were referenced with respect to the transcription of the *16S* gene from *P. aeruginosa*. One-way Analysis of Variance (ANOVA) with Tukey post-hoc test was used to compare the data. The different letters above the bars indicate statistical significance, values with identical letter were not considered significantly different ($p < 0.05$).

Figure 5. Binding of PumB and the PumA/PumB complex to the 5' region of the *pumAB* operon. **(a)** Schematic representation of the fragments used in the EMSA assays located at the 5' region of the *pumAB* operon and the *pumB* gene. Coding regions of *pumA* and *pumB* genes of the PumAB TA system from the pUM505 plasmid are indicated by large arrows, and the location of putative promoter sequences is marked by bent vertical arrows. DNA regions (thin lines) PpumAB, PpumAB', □PpumAB, PpumB/PpumB-AlgU and □PpumB/PpumB-AlgU used for EMSA and its size (in kb) are indicated. The oligonucleotides (small arrows) used for the amplification by PCR of the DNA fragments are indicated. **(b)** Purified DNA fragments (200 ng) containing the PpumAB region and the PchrS region were each mixed and incubated with the indicated amounts of the purified PumB-His protein as described in the Methods section. **(c)** Purified DNA fragments (200 ng) containing

the region indicated (PpumAB, $\square$ PpumAB, PpumB/PpumB-AlgU, or $\square$ PpumB/PpumB-AlgU) were each mixed and incubated with the indicated amounts of the purified PumB-His protein (μ g). **(d)** Purified DNA fragment (200 ng) containing the PpumAB' region was mixed and incubated with the indicated amounts of PumB-His and His-PumA purified proteins (μ g). The control of PpumAB region by gel retardation, with or without PumB-His protein is shown. **(e)** Purified DNA fragments (200 ng) containing the PpumAB region and the $\square$ PpumAB' region were each mixed and incubated with the indicated amounts of the purified PumB-His protein as described in the Methods section. For EMSA, free DNA and protein-DNA complexes were resolved by 6% native PAGE and stained with ethidium bromide. M, Molecular size markers shown to the left in base pairs.

Figure 6. PumA improves the binding of PumB to the 5' region of the *pumAB* operon. Binding of 1.0 $\square$ g PumB-His purified protein (which was previously mixed with the indicated amounts of His-PumA protein (μ g) to the purified DNA fragment (200 ng) containing the PpumAB region of the *pumAB* operon. For EMSA, free DNA and protein-DNA complexes were resolved by 6% native PAGE and stained with ethidium bromide. M, Molecular size markers shown to the left in base pairs.

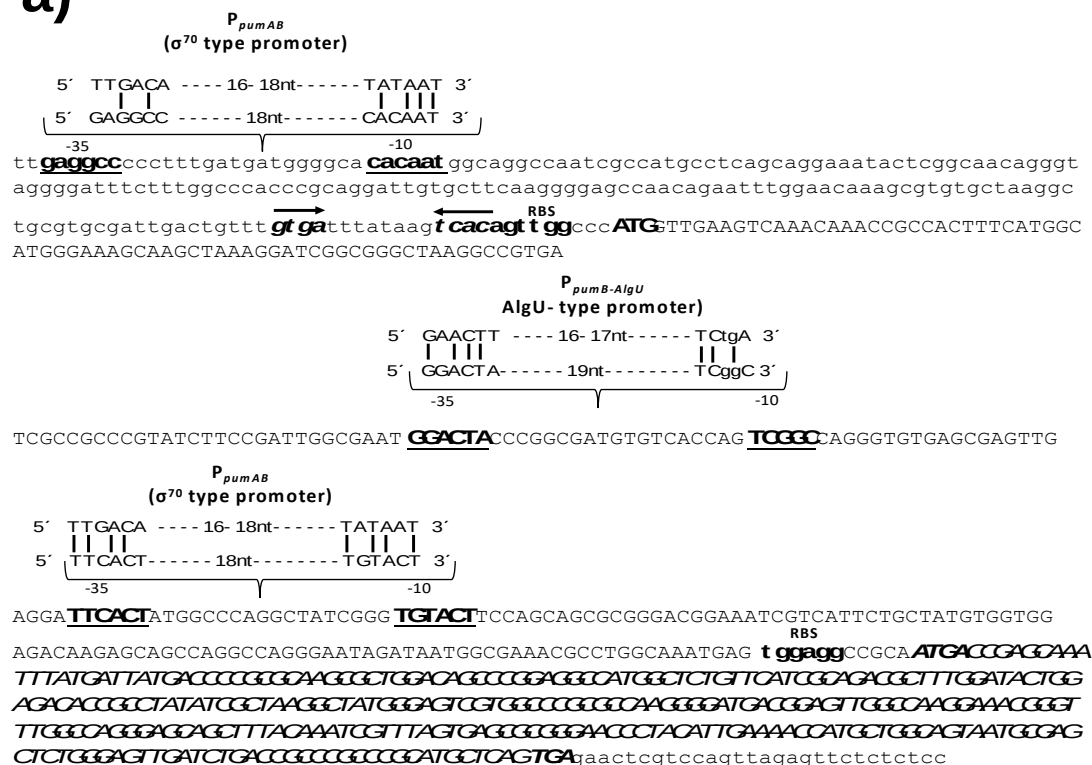
Figure 8. PumB protein decreases the stability of the *pumA* mRNA *in vitro*.

Total RNA (400 ng) isolated from cultures of the corresponding strain was incubated at 37°C in the absence and presence of 1.0 $\square$ g of PumB-His or His-PumA purified

protein. Later, the mRNA levels of *pumA* and *pumB* were determined by qRT-PCR as described in the Methods section. **(a)** The mRNA level of *pumA* from total RNA of *E. coli* BL21 (pTrcHis_*pumA*) after its incubation for 30 min. **(b)** The mRNA level of *pumA* after incubation for the time periods indicated, with the PumB-His purified protein. **(c)** The mRNA level of *pumB* from total RNA of *P. aeruginosa* PAO1 (pUC_*pumB*) after its incubation for 30 min, without (grey bars) and with (bars with lines) PumB-His purified protein. A control (black bar) is shown with the mRNA level of *pumB* after its incubation in the buffer of the protein elution (RM) without the addition of PumB-His. **(d)** The mRNA level of *pumA* from total RNA of *E. coli* BL21 (pTrcHis_*pumA*) after its incubation for 30 min, without (white) and with (bars with lines) His-PumA purified protein. **(e)** The mRNA level of *pumA* gene from total RNA of *E. coli* BL21 (pTrcHis_*pumAB*) after its incubation for 30 min, 1 and 2 h without (full bars) and with (bars with lines) PumB-His purified protein. **(f)** The mRNA level of *pumB* gene from total RNA of *E. coli* BL21 (pTrcHis_*pumAB*) after its incubation for 30 min, 1 and 2 h without (full bars) and with (bars with lines) PumB-His purified protein. **(g)** The mRNA level of the *pumA* gene in total RNA of *E. coli* BL21 (pTrcHis_*pumA*) incubated for 30 min after addition of the PumB-His protein, in the absence (bars with lines) and presence (bars with crossed lines) of 0.5, 1.0 and 2.0 μ L ribonuclease RNase OUT. As control, the mRNA level of *pumA* is shown, after 30 min of incubation in the buffer of the protein elution (RM) without PumB-His addition (white), and with PumB-His addition (black). Values were normalized with respect to the transcription of the *16S* genes from *E. coli* or *P. aeruginosa*. Values represent the means of three independent determinations in duplicate with standard error (SE) bars shown. One-way Analysis of Variance (ANOVA) with Tukey post-hoc

875 test was used to compare the data. The different letters above the bars indicate
876 statistical significance, values with identical letter were not considered significantly
877 different ($p < 0.05$).

a)



b)

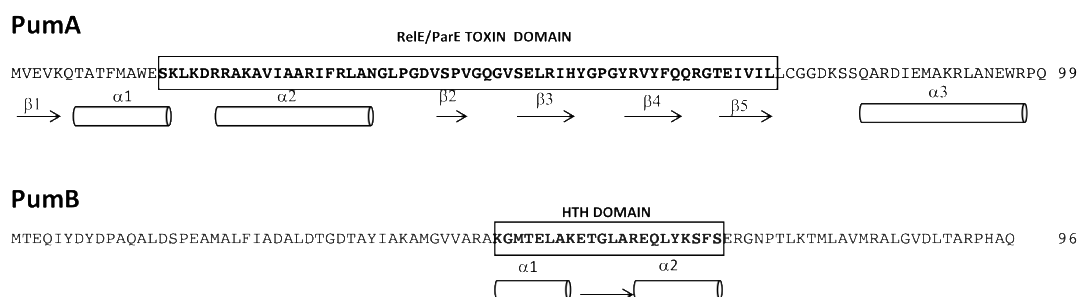
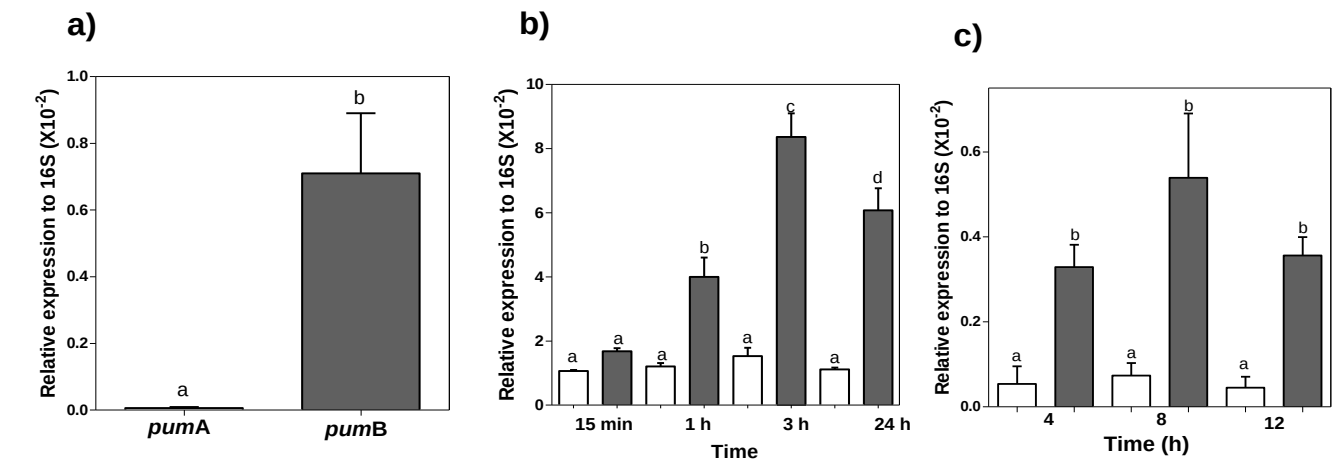


Figure 1.

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884 **Figure 2.**

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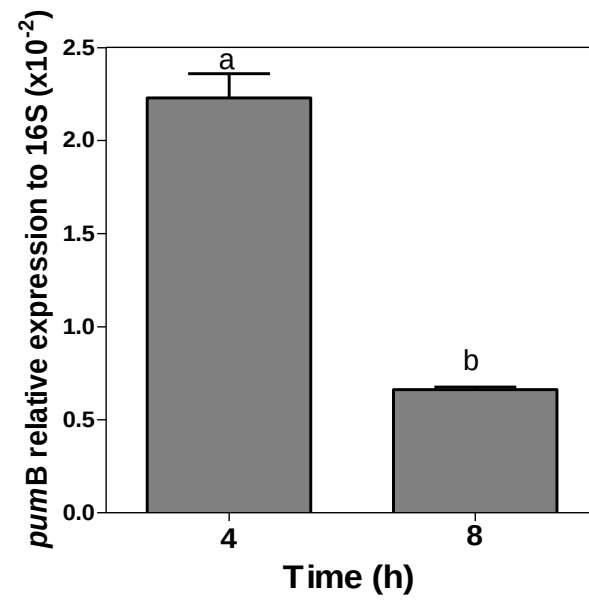


Figure 3.

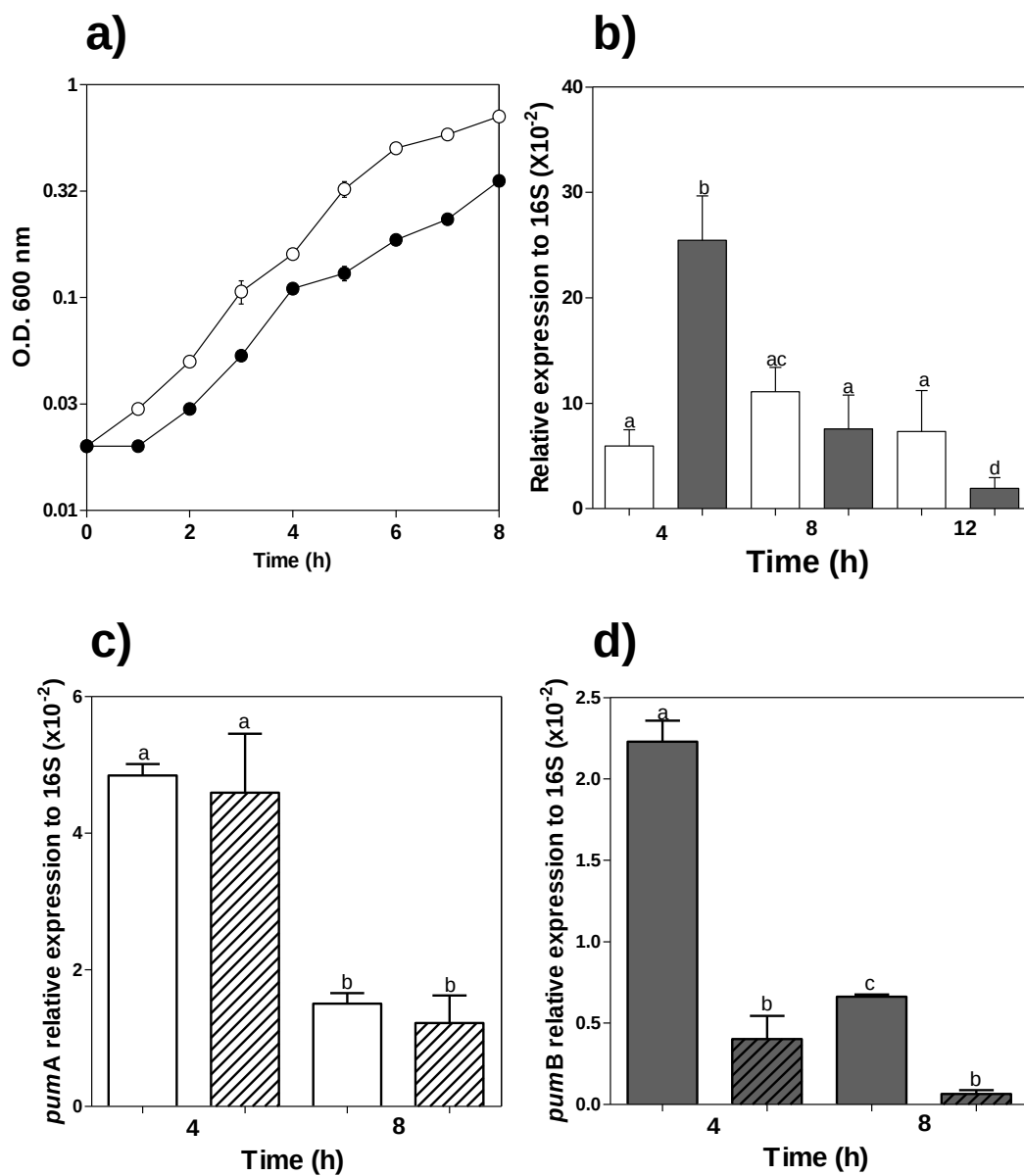


Figure 4.

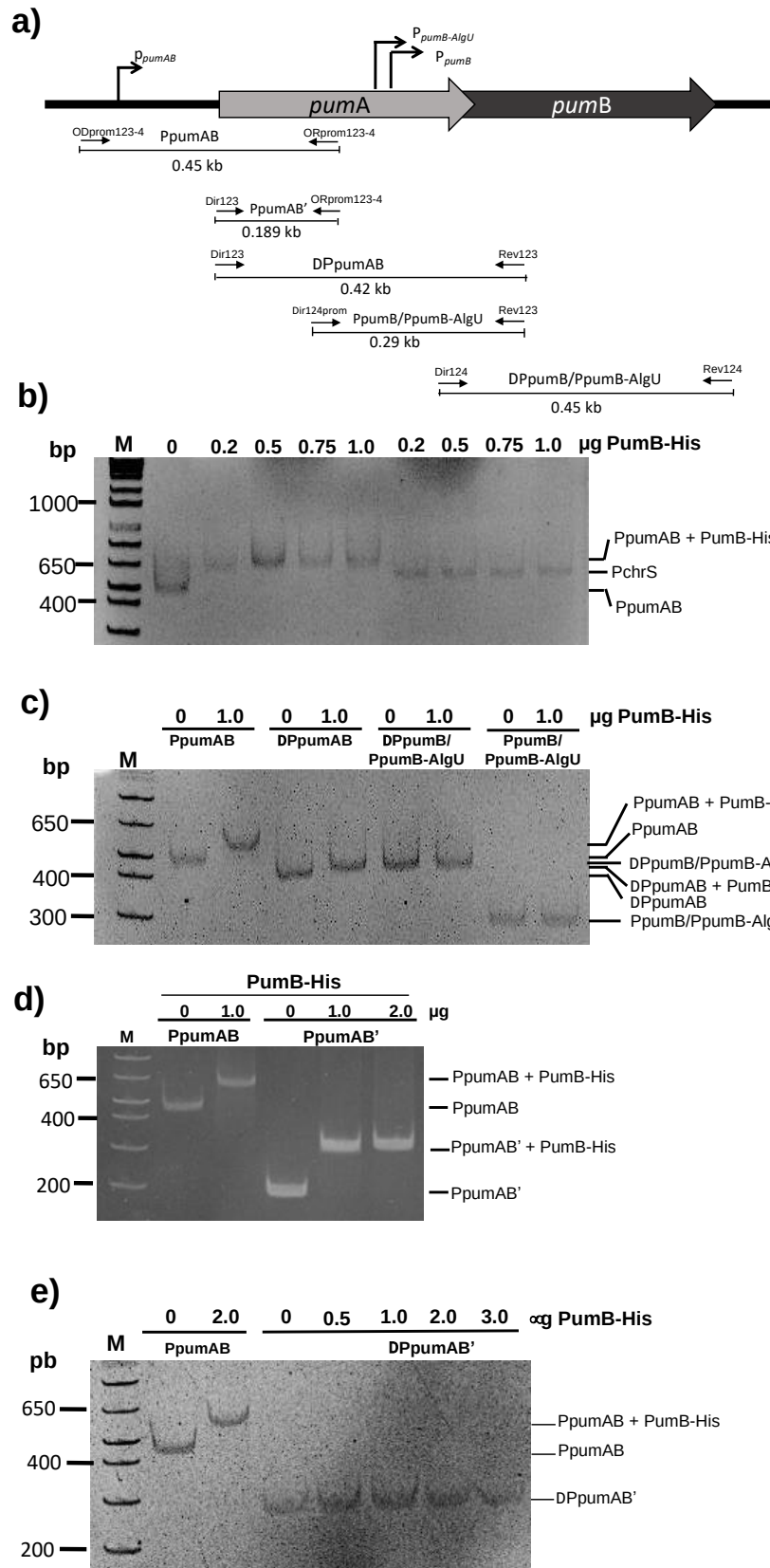
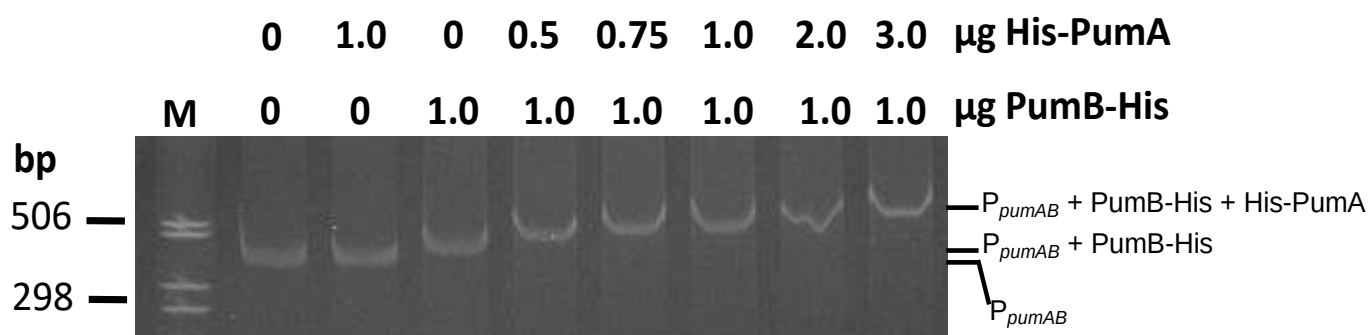


Figure 5.

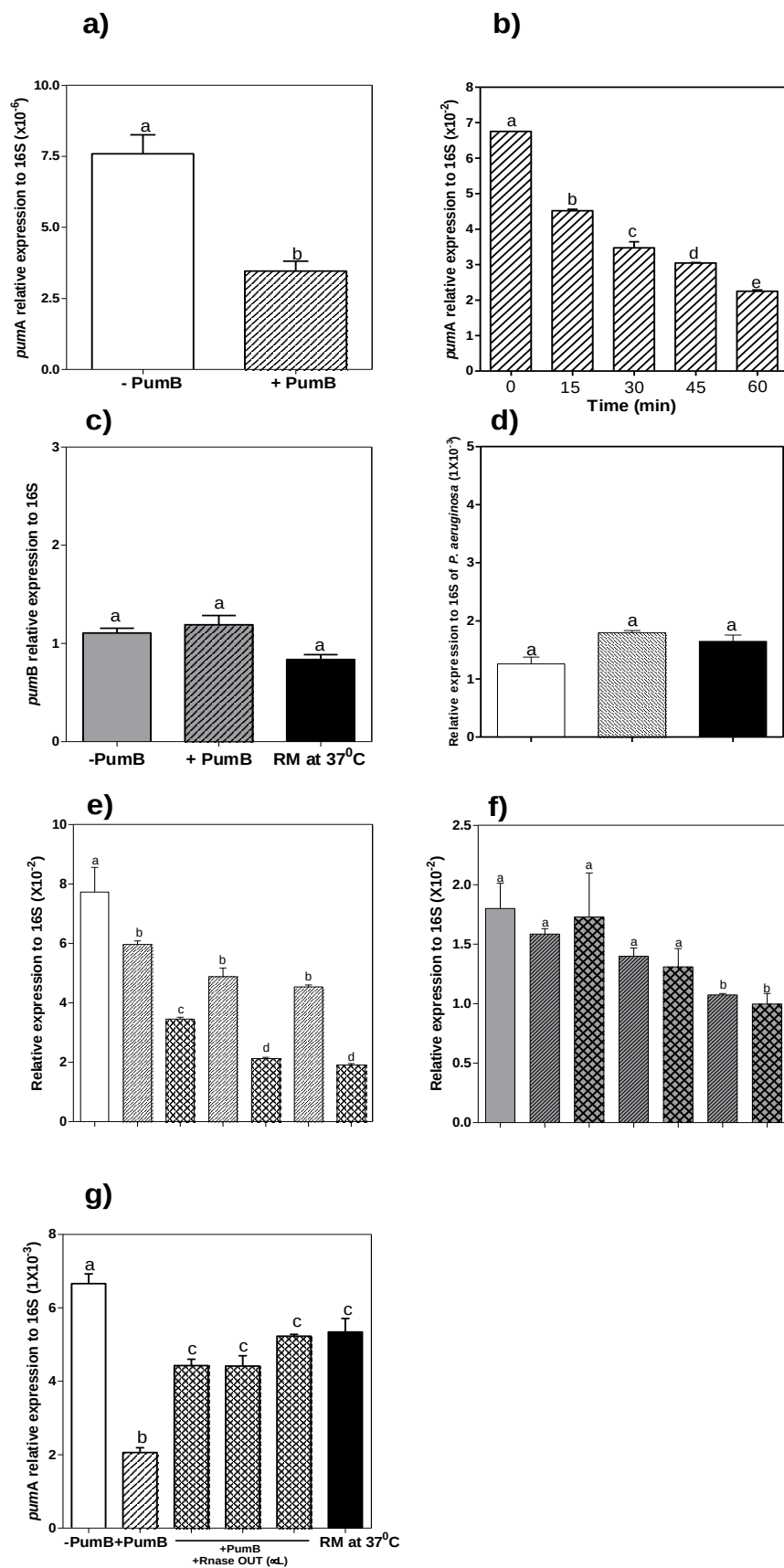
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931 **Figure 6.**



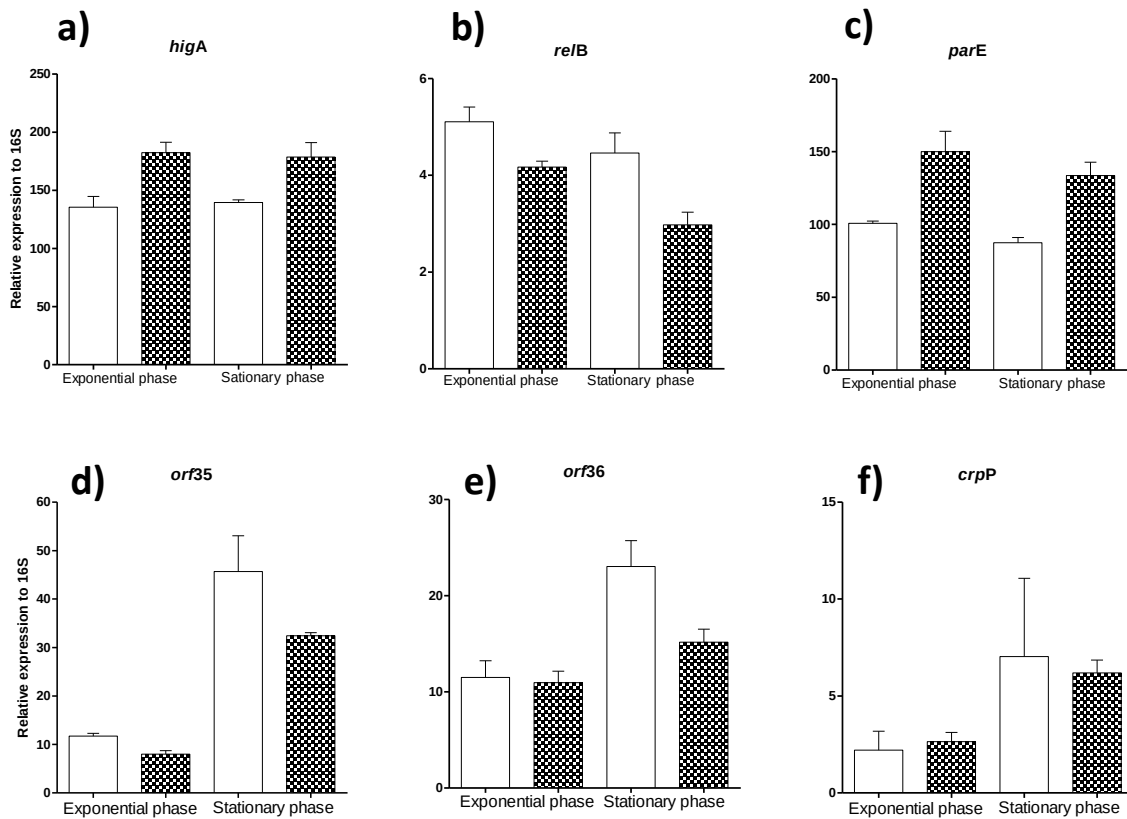


Figure 8.

CAPITULO III

Artículo: Virulence conferred by PumA toxin from the plasmid encoded PumAB

Toxin-Antitoxin System is Regulated by Quorum System.

Current Microbiology

VIRULENCE CONFERRED BY PumA TOXIN FROM THE PLASMID ENCODED PumAB TOXIN-ANTITOXIN SYSTEM IS REGULATED BY QUORUM SYSTEM –Manuscript Draft–

Manuscript Number:	CMIC-D-20-00016R2	
Full Title:	VIRULENCE CONFERRED BY PumA TOXIN FROM THE PLASMID ENCODED PumAB TOXIN-ANTITOXIN SYSTEM IS REGULATED BY QUORUM SYSTEM	
Article Type:	Original Paper	
Funding Information:	Coordinación de la Investigación Científica, UMSNH (2.6)	Not applicable
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Abstract:	Toxin-antitoxin (TA) systems are small genetic elements composed of a toxin gene and its cognate antitoxin that are important for plasmid stabilization (plasmid-encoded) and bacterial virulence (chromosome-encoded). These systems are also related to biofilm and persister cell formations. <i>Pseudomonas aeruginosa</i> is an antibiotic-resistant human pathogen that produces virulence factors modulated by quorum sensing (QS) and can form biofilms. The type II PumAB TA system of pUM505, isolated from a clinical strain of <i>P. aeruginosa</i>, confers plasmid stability. Additionally, the PumA toxin increases <i>P. aeruginosa</i> virulence and is neutralized by the PumB antitoxin. In this study, we determined whether virulence conferred by PumA toxin is regulated by QS. The <i>pumA</i> gene was transferred to <i>P. aeruginosa lasI/rhlI</i>, a mutant strain in the LasI and RhlI QS systems, to analyze the effect on virulence of the transformants. <i>pumA</i> transfer did not increase bacterial virulence in lettuce and <i>Caenorhabditis elegans</i>, suggesting that the virulence conferred by PumA requires QS modulation. <i>pumA</i> mRNA levels drastically decreased in the <i>P. aeruginosa lasI/rhlI</i> (pUC-<i>pumA</i>) strain, suggesting positive regulation of <i>pumA</i> gene expression by QS. Supplementation of the growth medium of <i>P. aeruginosa lasI/rhlI</i> (pUC-<i>pumA</i>) with C4-AHL and 3-oxo-C12-AHL autoinducers increased <i>pumA</i> mRNA levels and restored bacterial virulence, suggesting that both autoinducers complemented the mutations and positively regulated the toxic effects of PumA. This strengthened the hypothesis that QS regulates bacterial virulence conferred by the PumA toxin. Thus, this report establishes an important function of QS in the virulence conferred by plasmid-encoded TA systems in bacterial pathogens.	
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Author Comments:	June 5, 2020 Erko Stackebrandt Editor in Chief Current Microbiology Dear Editor: I wish to submit a Research Article for publication in Current Microbiology, titled "Virulence conferred by PumA toxin from the plasmid encoded PumAB toxin-antitoxin system is regulated by Quorum System". The paper was coauthored by Karen Cecilia Hernández-Ramírez, Brenda Valerio-Arellano, Marco Ivan Valle-Maldonado, León Francisco Ruiz-Herrera, Víctor Meza-Carmen and Martha I. Ramírez-Díaz, manuscript ID: CMIC-D-20-00016R1; which was put under revision recently suggesting incorporate minor revisions. In the new manuscript the changes suggested were made. The new manuscript was modified in order provide institutional information as well as the ORCID of the corresponding author. This study describes how the bacterial virulence conferred by the PumA toxin from the PumAB TA system, identified in the pUM505 plasmid from a clinical isolate of <i>Pseudomonas aeruginosa</i>, is regulated by the LasI and RhlI mechanism of Quorum system (QS). We also suggest that pumA expression is positive regulated by QS. Moreover, we demonstrate that the virulence is restored by the chemical addition of C4-AHL and 3-oxo-C12-AHL autoinducers of QS. We believe that our study makes a significant contribution to the literature because we describe that the QS is able to regulate the virulence conferred by a toxin (PumA) from a TA system plasmid-encoded. This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare. Thank you for your consideration. I look forward to hearing from you. Sincerely, Martha I. Ramírez Díaz Instituto de Investigaciones Químico-Biológicas Universidad Michoacana de San Nicolás de Hidalgo Edificio B-3, Ciudad Universitaria 58030 Morelia, Mich., México Phone/fax: 52+(443) 326-5788 E-mail: marthaisela_ramirez@hotmail.com
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**VIRULENCE CONFERRED BY P_{umA} TOXIN FROM THE PLASMID ENCODED
P_{umAB} TOXIN-ANTITOXIN SYSTEM IS REGULATED BY QUORUM SYSTEM**

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Abstract

Toxin-antitoxin (TA) systems are small genetic elements composed of a toxin gene and its cognate antitoxin that are important for plasmid stabilization (plasmid-encoded) and bacterial virulence (chromosome-encoded). These systems are also related to biofilm and persister cell formations. *Pseudomonas aeruginosa* is an antibiotic-resistant human pathogen that produces virulence factors modulated by quorum sensing (QS) and can form biofilms. The type II P_{um}AB TA system of pUM505, isolated from a clinical strain of *P. aeruginosa*, confers plasmid stability. Additionally, the P_{um}A toxin increases *P. aeruginosa* virulence and is neutralized by the P_{um}B antitoxin. In this study, we determined whether virulence conferred by P_{um}A toxin is regulated by QS. The *pumA* gene was transferred to *P. aeruginosa lasI/rhlI*, a mutant strain in the LasI and RhlI QS systems, to analyze the effect on virulence of the transformants. *pumA* transfer did not increase bacterial virulence in lettuce and *Caenorhabditis elegans*, suggesting that the virulence conferred by P_{um}A requires QS modulation. *pumA* mRNA levels drastically decreased in the *P. aeruginosa lasI/rhlI* (pUC_*pumA*) strain, suggesting positive regulation of *pumA* gene expression by QS. Supplementation of the growth medium of *P. aeruginosa lasI/rhlI* (pUC_*pumA*) with C4-AHL and 3-oxo-C12-AHL autoinducers increased *pumA* mRNA levels and restored bacterial virulence, suggesting that both autoinducers complemented the mutations and positively regulated the toxic effects of P_{um}A. This strengthened the hypothesis that QS regulates bacterial virulence conferred by the P_{um}A toxin. Thus, this report establishes an important function of QS in the virulence conferred by plasmid-encoded TA systems in bacterial pathogens.

Keywords: *P. aeruginosa*, toxin-antitoxin system, virulence, quorum system, PumAB system, PumA toxin.

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Authors' Contributions

KC H-R, B V-A and MI V-M, data acquisition, analysis, and data interpretation, LF R-H and V M-C, reagents, analysis; V M-C data interpretation, and critical revision of the article for intellectual content; MI R-D, conception and design of the work, data analysis and interpretation, and drafting of the article.

Conflicts of interest

The authors declare no conflict of interest.

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Introduction

Pseudomonas aeruginosa is one of the most significant opportunistic pathogens in humans.

It belongs to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter spp.*), which is a group of antibiotic-resistant bacteria that are common causes of life-threatening nosocomial infections [1]. Patients with chronic diseases or compromised immune systems due to immunodeficiency or burns are at a greater risk of *P. aeruginosa* infection [2]. The success of *P. aeruginosa* infections is largely due to the production of virulence factors that elicit disease in both plants and animals and the ability to form biofilms [3]. The production of several virulence factors by *P. aeruginosa* is controlled according to cell density via quorum-sensing (QS) systems. QS produces and releases small chemical signals, called autoinducers, which accumulate at a high population density and interact with cognate receptors to induce the transcriptional expression of various target genes, including those encoding virulence factor production [4]. The *P. aeruginosa* QS network is organized in a multi-layered hierarchy consisting of at least four interconnected signaling mechanisms, including *las*, *iqs*, *pqs*, and *rhl* [4]. The *las* and *rhl* systems regulate the production of multiple virulence factors as well as elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, and superoxidase dismutase [5].

Toxin-antitoxin (TA) systems are small genetic modules found in bacterial mobile genetic elements and bacterial chromosomes [6-7]. TA systems are prevalent in bacteria and archaea; although they are not essential for normal cell growth, they are implicated in multiple cellular functions associated with survival under stress conditions [8]. Plasmid-encoded TA systems are important for plasmid stabilization by a post-segregation killing mechanism that eliminates daughter cells that have lost the TA-encoding plasmid [9];

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4 chromosomally encoded TA systems are related to bacterial pathogen virulence [10-11].

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6 TA systems are also involved in biofilm formation and persister cell formation (persister
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8 cells are a small fraction of bacteria that demonstrate resistance to antibiotics without
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10 genetic change), suggesting that these systems may function in the stress response as
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12 regulators of the switch from the planktonic to the biofilm lifestyle [12]. A significant
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14 correlation between QS and TA systems in clinical *P. aeruginosa* isolates with a high
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16 biofilm formation ability has also been indicated [13].
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21 The PumA toxin has been shown to increase *P. aeruginosa* virulence effects neutralized by
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23 the PumB antitoxin [14]. Additionally, the supernatant from *P. aeruginosa* expressing
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25 PumA can kill *Caenorhabditis elegans*, suggesting that the toxin could be secreted into the
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27 media during bacterial growth. Moreover, purified PumA protein decreased *C. elegans*
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29 viability, demonstrating that PumA could act as a bacterial virulence factor [14]. The type
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31 II PumAB TA system of pUM505 has also been shown to confer plasmid stability to
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33 *Escherichia coli* [14].
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37 The aim of this work was to determine whether the virulence conferred by the PumA toxin
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39 from a TA system plasmid-encoded is regulated by QS. We report that QS regulates PumA
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41 toxicity and increases bacterial virulence. This establishes an important function for QS in
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43 the virulence conferred by plasmid-encoded TA systems in bacterial pathogens.
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50 **Materials and Methods**

51 **Bacterial strains, culture conditions, and plasmids**

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53 *P. aeruginosa* PAO1 (WT) and *P. aeruginosa* *rhII/lasI* double mutant (donated by Dr.
54
55 Jesús Campos García, Universidad Michoacana de San Nicolás de Hidalgo, México) were
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57 used as recipient strains for recombinant plasmids or virulence assays. The pUC_pumA,
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4 pUC_pumB, and pUC_pumAB recombinant plasmids contain the *pumA*, *pumB*, or *pumAB*
5
6 genes, respectively, and were cloned with their own promoters into the pUCP20
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8 *Escherichia/Pseudomonas* shuttle vector [14]. The recombinant plasmids were transferred
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10 to *Pseudomonas* strains by electroporation before selecting transformants in LB plates
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12 supplemented with carbenicillin (Sigma, St. Louis, MO, USA).
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14 The culture medium used was Luria-Bertani (LB) broth. 1.5% agar was added to form solid
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16 media [14]. M9 salts minimal medium (Sigma) supplemented with 20 mM glucose and 0.1
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18 mM CaCl₂. Carbenicillin (400 µg mL⁻¹) was added to agar plates when necessary. Cultures
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20 were routinely grown overnight at 37°C with shaking, and growth was monitored based on
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22 optical density (OD) at 600 nm (OD₆₀₀) using a spectrophotometer.
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31 Genetic techniques and sequence analysis

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33 Molecular genetics procedures were carried out using standard methods [15].
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37 Bacterial growth assays

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39 Liquid cultures of *P. aeruginosa* PAO1 or *P. aeruginosa lasI/rhlI* double mutants
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41 containing the recombinant plasmids were used to evaluate the effects of QS mutation on
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43 transformant growth. Overnight cultures were diluted 1:50 in 250 mL of fresh LB or M9
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45 medium, and growth was monitored at intervals by measuring OD₆₀₀.
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52 RNA isolation and RT-qPCR analysis

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54 To measure *pumA* and *pumB* gene expression in *P. aeruginosa* strains, cultures were
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56 incubated in the exponential and stationary phases of growth, and cells were harvested by
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58 centrifugation. Total RNA was isolated and quantified as previously described [14].
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Oligonucleotide primers and hydrolysis probes for RT-qPCR of the *pumA* gene and *16S* rRNA reference genes were described by Hernández-Ramírez et al. [14]. Amplification of *pumA* and *pumB* genes and *16S* rRNA genes was performed using the 5' exonuclease probe RT-qPCR method. RT-qPCR was performed with total RNA samples (500 ng) and the SuperScript III Platinum One-Step RT-qPCR Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) with the LightCycler 480 II System (Roche Molecular Diagnostics, Pleasanton, CA, USA). Amplification signal curves were analyzed at the absorption wavelength of 530 nm. Appropriate positive and non-template controls were included in each test run. Relative expression of the *pumA* and *pumB* genes was normalized to the expression values obtained from the *P. aeruginosa* *16S* rRNA gene (accession number AE004091; region: 722096–723631). Relative gene expression was estimated using the classical calibration dilution curve and slope calculation. A five-fold dilution series (500–0.05 ng total RNA) was prepared and used as the sample in the RT-qPCR. Efficiency (*E*) was obtained from standard curves using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$. For chemical complementation, the *P. aeruginosa* bacterial cultures were supplemented with 0.6 μM of N-decanoyl-DL-homoserine lactone (C4-AHL) (Fluka, USA) or N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL) (Sigma) before growing overnight at 37°C to the exponential phase. Total RNA extraction and RT-qPCR were performed as described previously. Relative expression levels were determined using the efficiency correction method, which considers amplification efficiencies between target and reference genes [16].

Virulence assays

Lettuce-leaf model of infection

P. aeruginosa infection on lettuce leaves was performed as previously described [17]. Cultures were grown overnight in LB broth at 37°C. After centrifugation, the pellets were suspended in fresh LB broth and grown for an additional 4 h. The resulting log-phase cultures were diluted with 10 mM MgSO₄ to an OD₆₀₀ of 0.2. Lettuce midribs were washed with distilled H₂O and 0.1% (v/v) bleach before inoculating using pipette tip puncture. We inoculated 1×10^6 ($\pm 1 \times 10^4$) colony-forming units (CFU) in 10 μ L of the bacterial suspensions onto lettuce leaves, which were placed on Whatman filter paper moistened with 10 mM MgSO₄. Symptoms were monitored daily, and the length and width of the lesions were measured. The lesion area was estimated as previously described [18]. The experiments were repeated at least three times. The number of CFUs present within a lettuce lesion was determined using a procedure adapted from Dong et al. [19]. Briefly, after 4 days of incubation, the infected lettuce leaf region was cut from the midrib and macerated in 5 mL of 10 mM MgSO₄ with a mortar and pestle. Serial dilutions of the suspension in 10 mM MgSO₄ were plated on LB agar to count the number of bacterial CFUs after incubation at 37°C for 24 h. Bacterial inoculum concentrations were confirmed for all assays using dilution plating.

C. elegans killing assays

C. elegans Bristol N2 [20] worms were synchronized using hypochlorite isolation of eggs from gravid adults before hatching the eggs in S-basal medium [21]. L1 larvae were transferred to NGM (Nematode Growth Medium) [21] in plates seeded with the *E. coli* OP50 strain previously grown on the plates as a food source and incubated at 20°C for 4–5

days until they reached the young adult phase. Worms were rinsed off the plates and washed in S-basal medium. Cultures of *P. aeruginosa* strains were grown overnight in M9 broth at 37°C, centrifuged, and the pellets were suspended in fresh M9 broth and grown to an OD₆₀₀ of 0.4. To chemically complement *P. aeruginosa* *rhII/lasI* mutant bacterial cultures in M9 were supplemented with 0.6 μM of N-decanoyl-DL-homoserine lactone (C4-AHL) (Fluka, USA), N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL) (Sigma) or N-decanoyl-DL-homoserine lactone (C10-AHL) (Fluka) before growing overnight at 37°C and centrifuged. The pellets were suspended in fresh M9 broth and grown to an OD₆₀₀ of 0.4. For each experiment, 20 worms were dispensed into each well of a 24-well Costar plate (Corning, Inc., Corning, NY, USA). The worms were incubated with 1×10^5 ($\pm 1 \times 10^4$) CFU of the bacterial strain contained in a total volume of 1 mL. The plates were incubated for 36 h at 20°C and scored for live worms at 6, 12, 24, and 36 h. *P. aeruginosa* PAO1 (pUC_pumA) and M9 medium were used as positive and negative controls, respectively. A worm was considered dead when it no longer responded (moved) to a touch stimulus. Worms that died as a result of adhering to the plate wall were excluded from the analysis. For all assays, bacterial concentrations were confirmed by dilution, plating, and counting. Two independent assays with two replicates were conducted for each worm group.

Statistical analysis

Data analysis was performed using GraphPad Prism 5 software. Analysis of variance (ANOVA) was performed for all the *Pseudomonas* assays. $P < 0.05$ was considered statistically significant.

Results

QS regulates the virulence of *P. aeruginosa* conferred by *pumA*

The PumA toxin of the PumAB TA system increases *P. aeruginosa* virulence [14]. To determine whether the virulence ability of PumA depends on QS, the recombinant plasmids with *pumA* and *pumAB* genes were transferred to *P. aeruginosa lasI/rhlI* (double mutant) and virulence assays were conducted. First, virulence conferred by *pumAB* genes was analyzed using the lettuce-leaf model. The *P. aeruginosa* PAO and *P. aeruginosa lasI/rhlI* strains were transformed with the pUCP20 vector and virulence assays were conducted to discount the possibility that the vector backbone/antibiotic selection marker affects the virulence of the strains; the results showed no significant difference in CFU counts between the strains (Fig. 1). CFUs increased by 69% when the lettuce was inoculated with *P. aeruginosa* PAO1 (pUC_pumA) compared to when the lettuce was inoculated with *P. aeruginosa* PAO1 (pUCP20), empty vector only (Fig. 1). This indicated that PumA increased bacterial virulence in lettuce. Moreover, *P. aeruginosa* PAO1 (pUC_pumAB) decreased the number of CFUs, with values similar to those of the *P. aeruginosa* PAO1 (pUC_pumB) strain. This indicated that, as described [14], PumB neutralizes the toxic PumA effects and does not participate in the bacterial virulence (Fig. 1). The *P. aeruginosa lasI/rhlI* mutant had approximately 82% fewer CFUs in lettuce than *P. aeruginosa* PAO1. This result was expected because several *P. aeruginosa* virulence factors depend on QS function (Fig. 1). However, the *P. aeruginosa lasI/rhlI* (pUC_pumA) strain did not differ in terms of the number of CFUs compared to the *P. aeruginosa lasI/rhlI* (pUCP20), *P. aeruginosa lasI/rhlI* (pUC_pumB), and *P. aeruginosa lasI/rhlI* (pUC_pumAB) strains (Fig. 1). This suggested that the virulence conferred by PumA is LasI/RhlI QS dependent. Additionally, growth kinetics assays were performed to test the possibility that virulence

changes were due to growth rate differences in the strains analyzed; these showed no notable variations in growth among the strains (Fig. S1), suggesting that the lack of virulence is due to PsmA QS dependence. *P. aeruginosa* containing the *pumA* gene, and also purified PsmA toxin alone, has been reported to be toxic to *C. elegans* [14]; therefore, the ability of *pumA* to kill *C. elegans* in the *P. aeruginosa lasI/rhlI* mutant was assessed. We determined that *P. aeruginosa* PAO and *P. aeruginosa lasI/rhlI* strains transformed with the pUCP20 vector were non-virulent to *C. elegans*, as nematodes survival was the same as on M9 medium only (Fig. S2). This indicates again that the vector backbone/antibiotic selection marker does not affect the strain virulence. *P. aeruginosa lasI/rhlI* (pUC_pumA) was non-virulent to *C. elegans* compared with the *P. aeruginosa* PAO1 (pUC_pumA) strain, which decreased *C. elegans* survival by more than 45% (Fig. 2). Additionally, nematodes incubated with *P. aeruginosa lasI/rhlI* (pUC_pumA) showed the same survival values (around 85%) as those incubated only in M9 medium or with the *P. aeruginosa* PAO1 and *P. aeruginosa lasI/rhlI* strains (Fig. 2 and Fig. S2). These results strengthen the hypothesis that PsmA function is QS dependent.

QS positively regulates *pumA* mRNA levels

We determined *pumA* gene expression levels using RT-qPCR assays using RNA from *P. aeruginosa lasI/rhlI* (pUC_pumA) and compared them with the levels obtained for the *P. aeruginosa* PAO1 (pUC_pumA) strain. The *pumA* mRNA was approximately 8 and 4 times less abundant in *P. aeruginosa lasI/rhlI* (pUC_pumA) cultures at exponential and stationary growth phases, respectively, than in the *P. aeruginosa* PAO1 (pUC_pumA) cultures (Fig. 3a). This suggested *pumA* gene expression was positively regulated by QS. To analyze whether QS regulates *pumA* gene mRNA levels when its gene forms part of the

pumAB operon, *pumA* mRNA levels from *P. aeruginosa lasI/rhlI* (pUC_ *pumAB*) were compared with levels from *P. aeruginosa* PAO1 (pUC_ *pumAB*). These results were similar to those for *pumA* cloned without *pumB*: the mRNA levels of *pumA* decreased 11 and 13 times in cultures from exponential and stationary growth phases of *P. aeruginosa lasI/rhlI* (pUC_ *pumAB*), respectively, compared to the levels in *P. aeruginosa* PAO1 (pUC_ *pumAB*) (Fig. 3). This suggested that the *pumAB* operon was regulated by QS. Although *pumA* mRNA levels decreased in the *P. aeruginosa lasI/rhlI* mutant strain, *pumA* transcription was detected, suggesting that this gene can be modulated by another QS mechanism or condition of growth. The *pumB* mRNA levels for *P. aeruginosa lasI/rhlI* (pUC_ *pumAB*) were determined to see whether the *pumB* transcript is also regulated by QS. The results did not show differences in *pumB* mRNA levels in cultures from exponential phase growth of *P. aeruginosa lasI/rhlI* (pUC_ *pumAB*) compared to the levels of *P. aeruginosa* PAO1 (pUC_ *pumAB*). However, *pumB* transcript levels decreased approximately 3.5 times in cultures from the *P. aeruginosa lasI/rhlI* (pUC_ *pumAB*) strain compared to those from cultures of *P. aeruginosa* PAO1 (pUC_ *pumAB*) (Fig. 3c). This supports the hypothesis that QS regulates *pumAB* operon expression.

Chemical complementation of the *P. aeruginosa rhII/lasI* (pUC_ *pumAB*) strain with C4-AHL and 3-oxo-C12-AHL increased the mRNA levels of *pumA* and restored the virulence conferred by the toxin

3-oxo-C12-AHL and C4-AHL are QS autoinducers in *P. aeruginosa*. On binding to their respective receptors, the proteins LasR and RhlR are activated and form complexes that bind to the conserved *las-rhl* boxes in the promoters of target genes and activate their expression [22]. To evaluate whether the expression of *pumA* through QS is regulated by

the autoinducer molecules, we added 3-oxo-C12-AHL and C4-AHL to the culture medium of *P. aeruginosa lasI/rhlI* (pUC_pumA) to complement the *lasI/rhlI* mutation and determined the mRNA levels of *pumA*. The addition of both autoinducers increased the expression of *pumA* in *P. aeruginosa lasI/rhlI* (pUC_pumA) in comparison with the absence of autoinducers (Fig. 4) and was more similar to the mRNA expression of the *P. aeruginosa* PAO1 (pUC_pumA) strain; addition of AHLs also increased *pumA* mRNA levels in the *P. aeruginosa* PAO1 (pUC_pumA) (Fig. 4). These results add weight to the hypothesis of positive regulation of *pumA* gene expression by QS.

To demonstrate that the increase in *pumA* expression, via 3-oxo-C12-AHL and C4-AHL complementation of the *lasI/rhlI* mutation, restored QS-mediated PmnA toxin bacterial virulence, we determined the virulence of *P. aeruginosa lasI/rhlI* (pUC_pumA) in *C. elegans*. The addition of both AHLs to *P. aeruginosa* PAO1 (pUC_pumA) did not improve the bacterial ability to kill *C. elegans* (Fig. 5a). Interestingly, the addition of 3-oxo-C12-AHL and C4-AHL increased *P. aeruginosa lasI/rhlI* (pUC_pumA) virulence (Fig. 5b). However, 3-oxo-C12-AHL clearly restored *P. aeruginosa lasI/rhlI* (pUC_pumA) virulence to a greater degree, as its addition limited nematode survival to approximately 48% compared to approximately 71% survival in *C. elegans* obtained under C4-AHL complementation. This suggests that virulence conferred by PmnA may respond better to the QS LasI system than the RhlI system. In addition, chemical complementation of the double *rhlI/lasI* mutant by 3-oxo-C12-AHL and C4-AHL revealed that virulence by *P. aeruginosa* PmnA toxin is likely controlled by the LasI and RhlI QS systems.

Interestingly, it has been described that the nosocomial and soil-based *Acinetobacter* species use different signal molecules in bacterial communication. Soil-borne isolates can produce 3-oxo-C8-AHL and C8-AHL, but nosocomial-derived isolates can produce longer-

chain signals such as C10-AHL, C12-AHL, C14-AHL, and C16-AHL [23]. In addition, in *P. aeruginosa* clinical isolates, C4-AHL was not detected in the *P. aeruginosa* PAO1 strain, suggesting that the clinical isolates either failed to make short-chain AHLs or the level of the signal molecules was very low [24]. The PumA toxin was identified in pUM505 isolated from a *P. aeruginosa* clinical strain [25], and we therefore determined whether the PumA function could be regulated by other AHL compounds in addition to 3-oxo-C12-AHL and C4-AHL. N-decanoyl-L-homoserine lactone (C10-AHL) was added to the *P. aeruginosa lasI/rhlI* (pUC_pumA) cultures and increased the virulence of this strain, with only 53% nematode survival. This was similar to the lethality percentage in *C. elegans* when 3-oxo-C12-AHL was added (Fig. 5c). This suggests that PumA toxin function could also be modulated by different QS long-chain signal molecules.

Discussion

TA systems are found in bacterial mobile genetic elements and bacterial chromosomes. They are linked to many aspects of cell physiology, including plasmid maintenance, stress response, persistence, protection from phage infection, and biofilm formation [12, 26]. The type II PumAB TA system confers plasmid stability, as well as other plasmid-encoded TA systems, and could participate in bacterial virulence via the function of its PumA toxin, such as chromosomally encoded TA systems. The transference of *pumA* to the *P. aeruginosa* PAO1 strain increased bacterial virulence, suggesting that the encoded protein could be a virulence factor [14]. Here, we explored whether QS is involved in the virulence conferred by the PumA toxin from the PumAB plasmid-encoded TA system and evaluated the effects of *pumA* gene in the *P. aeruginosa lasI/rhlI* double mutant. We found that PumA did not increase *P. aeruginosa lasI/rhlI* virulence in lettuce or against *C. elegans*.

This is contrary to what was observed in *P. aeruginosa* PAO1, indicating that the virulence conferred by PumA is QS-dependent. In addition, it was observed that in *P. aeruginosa* PAO1 the transference of *pumB* and *pumAB* genes decreased the bacterial virulence, we do not have an explication of this result; however, this suggests that the PumB antitoxin could, in some way, protect the bacteria from virulence factors and more analyses are required to determine this effect. QS has been shown to control virulence gene expression in gram-negative and gram-positive bacteria, and in some cases, this phenomenon has proven to be relevant for bacterial virulence *in vivo* [27]. *P. aeruginosa* QS systems play important roles in physiological and metabolic behaviors such as virulence, antibiotic resistance, motility, and biofilm formation [13]. Interestingly, in addition to QS mechanisms that promote biofilm maturation [26], multiple TA systems are involved in human pathogenic bacteria biofilm formation [12, 28-29]. Our data suggest that the PumA toxin acts as a virulence factor that could be regulated by QS. This strengthened the hypothesis of a correlation between QS and TA systems.

In addition, our data showed that *pumA* mRNA levels drastically decreased in the *P. aeruginosa lasI/rhlI* mutant background, suggesting that *pumA* expression is regulated by QS. In *P. aeruginosa*, QS plays a key role in modulating virulence gene expression, and the Las and Rhl systems regulate the timing and production of multiple virulence factors such as elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, and superoxidase dismutase [27]. The decrease in the *pumA* transcript in the *P. aeruginosa rhlI/lasI* mutant background could explain why *pumA* failed to increase the virulence of this strain in both lettuce and *C. elegans*. The *pumA* and *pumB* genes are co-transcribed as a bicistronic mRNA, similar to most genes encoded in TA systems, suggesting that they form an operon [14]. The RT-qPCR results showed that *pumA* mRNA levels also decreased

when *pumB* was expressed in the *P. aeruginosa lasI/rhlI* strain. Moreover, the *pumB* transcript was also decreased in the *P. aeruginosa rhlI/lasI* mutant. This suggests that the QS system regulates the *pumAB* operon. In addition, it was observed that the *pumA* mRNA levels also decreased the *P. aeruginosa lasI/rhlI* mutant compared to the *P. aeruginosa* PAO1 background, independent of the bacterial growth phase. This result is not surprising since evidence suggests that although QS occurs at high cell density in many microorganisms, it may play a more central role in the physiology of bacteria, where QS pathways converge with starvation-sensing pathways to regulate cell entry into stationary phase [30].

Several QS systems found in many bacterial pathogens produce and release small chemical signals called autoinducers, which are commonly associated with the regulation of genes encoding virulence factor production and biofilm formation [31-34]. The *lasI* gene in *P. aeruginosa* synthesizes 3-oxo-C12-AHL, an acyl homoserine lactone signal (autoinducer), and the *rhlI* gene synthesizes C4-AHL, a second autoinducer [22]. Chemical complementation by addition of 3-oxo-C12-AHL and C4-AHL compounds to *P. aeruginosa lasI/rhlI* (pUC_*pumA*) cultures increased the mRNA levels of *pumA* and restored bacterial virulence in *C. elegans*, indicating that both LasI and RhlI systems regulate PumA function. Although both autoinducers restored virulence, 3-oxo-C12-AHL addition restored *P. aeruginosa lasI/rhlI* (pUC_*pumA*) virulence to a greater degree than C4-AHL addition, and also induced higher *pumA* mRNA levels than C4-AHL. A study in *P. aeruginosa lasI* and *rhlI* mutants revealed that some genes respond well to 3-oxo-C12-AH (e.g., *lasA*), while other genes responded well with C4-AHL (e.g., *rhlAB*), and some equally well to both signals [35-36]. Our data suggested that virulence conferred by PumA responds better to the QS LasI system than the RhlI system.

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In summary, this work describes for the first time the role of QS in the regulation of
virulence conferred by toxins from plasmid-encoded TA systems in bacterial pathogens.

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Figure legends

Figure 1. Virulence conferred by PumA toxin in the *P. aeruginosa lasI/rhlI* double mutant in a lettuce leaf infection model. Colony forming units (CFU) were determined from the lesion areas of lettuce leaves infected with bacterial strains. CFU were quantified as described in the methods section. Data represent the means of six independent experiments with standard deviation (SD). Statistically significant differences are indicated by letters (Tukey's test).

Figure 2. Effect of *pumA* expression in the *P. aeruginosa lasI/rhlI* double mutant on *Caenorhabditis elegans* survival. Effects of *P. aeruginosa* PAO1 and *P. aeruginosa lasI/rhlI* transformants with *pumA* gene on *C. elegans* viability. Groups of 20 worms were incubated with $1 \times 10^5 (\pm 1 \times 10^4)$ colony-forming units (CFU) of the indicated strains as described in the methods. Mixtures were incubated at 20°C and scored for live worms at the indicated times. The data shown are the means from the duplicates of four independent assays.

Figure 3. *pumA* gene expression regulation by the Las and Rhl QS systems. Total RNA (500 ng) from cultures was used to determine the expression levels of the *pumA* or *pumB* genes using RT-qPCR as described in the methods. (a) Expression of *pumA* from *P. aeruginosa* PAO1 (pUC_*pumA*) (white) and *P. aeruginosa lasI/rhlI* (pUC_*pumA*) (gray) cultures grown at the exponential and stationary phases. Expression of *pumA* (b) and *pumB* (c) genes from cultures of *P. aeruginosa* PAO1 (pUC_*pumAB*) (white), and *P. aeruginosa lasI/rhlI* (pUC_*pumAB*) (gray) grown at the exponential and stationary phases. Values represent the means of three independent determinations in duplicate with standard error bars

shown. Data were normalized with respect to the transcription of the 16S gene from *P. aeruginosa*. Significant differences ($p < 0.0001$) are indicated by the letters.

Figure 4. Chemical complementation with AHLs increased *pumA* mRNA levels in the *P. aeruginosa* *rhII/lasI* mutant. Total RNA (500 ng) from cultures was used to determine the expression levels of *pumA* using RT-qPCR as described in the methods. Expression of *pumA* from *P. aeruginosa* PAO1 (pUC_*pumA*) and *P. aeruginosa* *lasI/rhII* (pUC_*pumA*) cultures grown with or without C4-AHL and 3-oxo-C12-AHL addition. Values represent the means of two independent determinations in duplicate with standard error bars shown. Data were normalized with respect to the transcription of the 16S gene from *P. aeruginosa*. Significant differences ($p < 0.0001$) are indicated by the letters.

Figure 5. Restoration of virulence conferred by PumA toxin using chemical complementation with AHLs in the *P. aeruginosa* *rhII/lasI* double mutant. Groups of 20 worms were incubated with $1 \times 10^5 (\pm 1 \times 10^4)$ colony-forming units (CFU) of each strain as described in the Methods section. (a) *C. elegans* viability after incubation with *P. aeruginosa* PAO1 or *P. aeruginosa* PAO1 (pUC_*pumA*) with or without C4-AHL, 3-oxo-C12-AHL, and C10-AHL addition. (b) *C. elegans* viability after incubation with *P. aeruginosa* *lasI/rhII* or *P. aeruginosa* *lasI/rhII* (pUC_*pumA*) with or without C4-AHL and 3-oxo-C12-AHL addition. (c) Effect of C10-AHL addition on *P. aeruginosa* *lasI/rhII* virulence conferred by PumA. Mixtures were incubated at 20°C and scored for live worms at the indicated times. The data shown are the means from the duplicates of four independent assays.

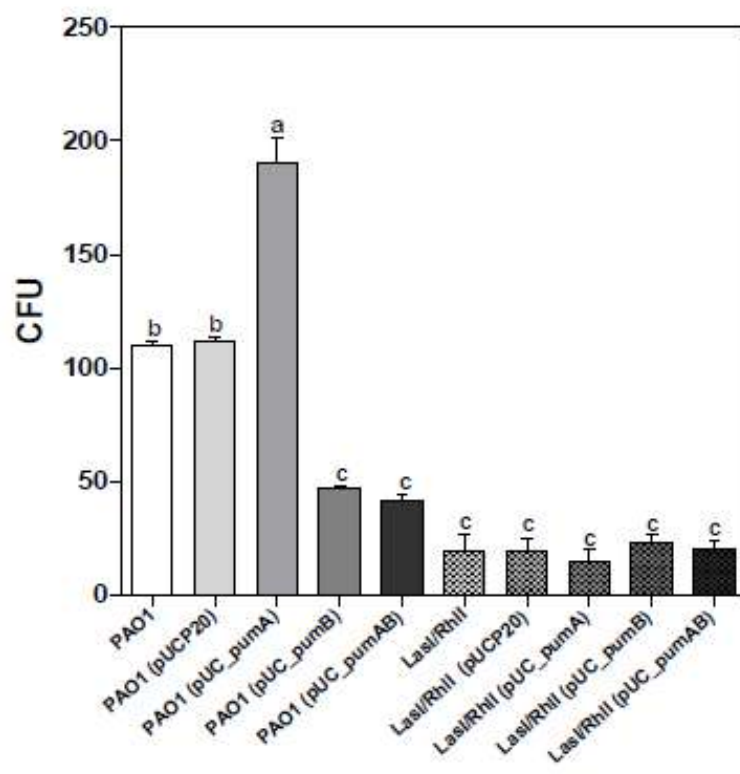


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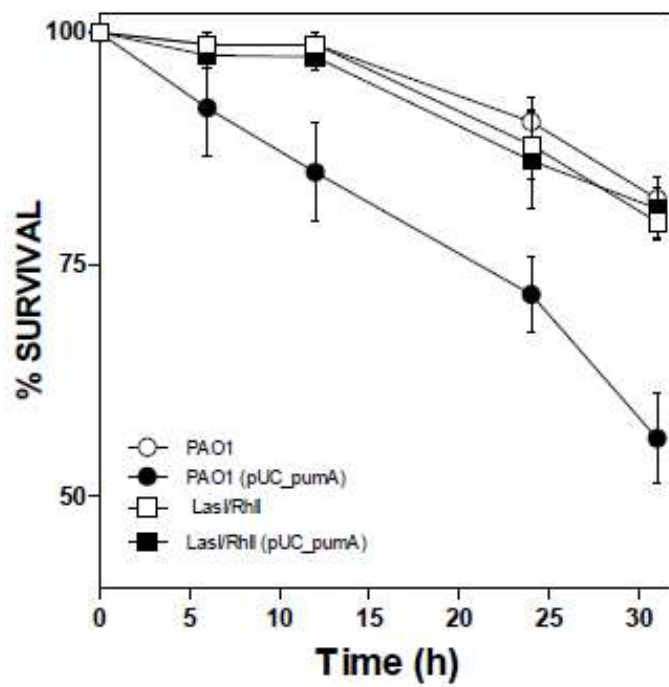


Figure 2.

Figure 3

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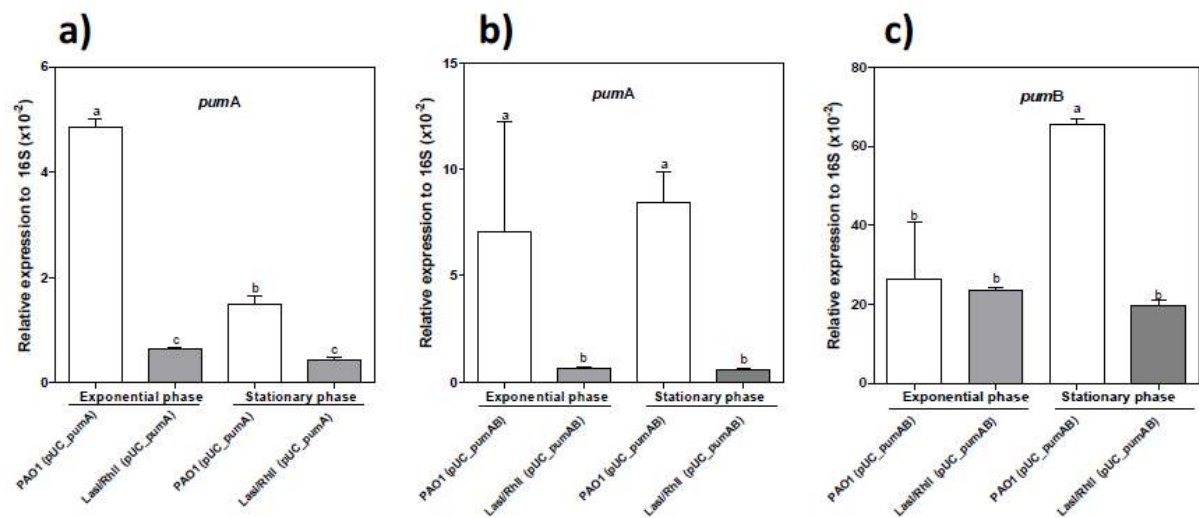


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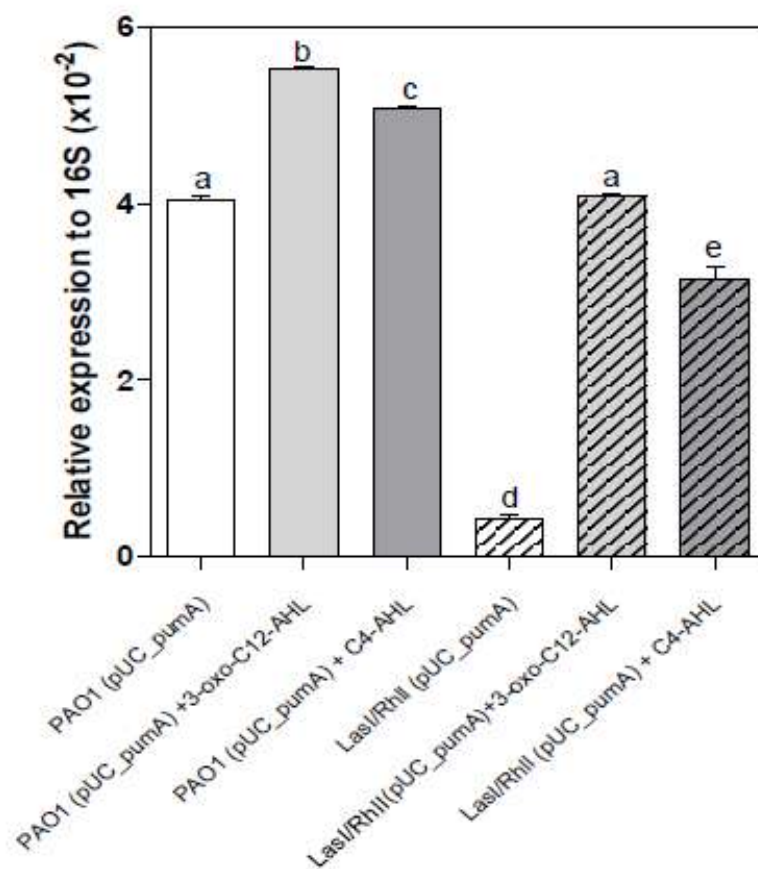


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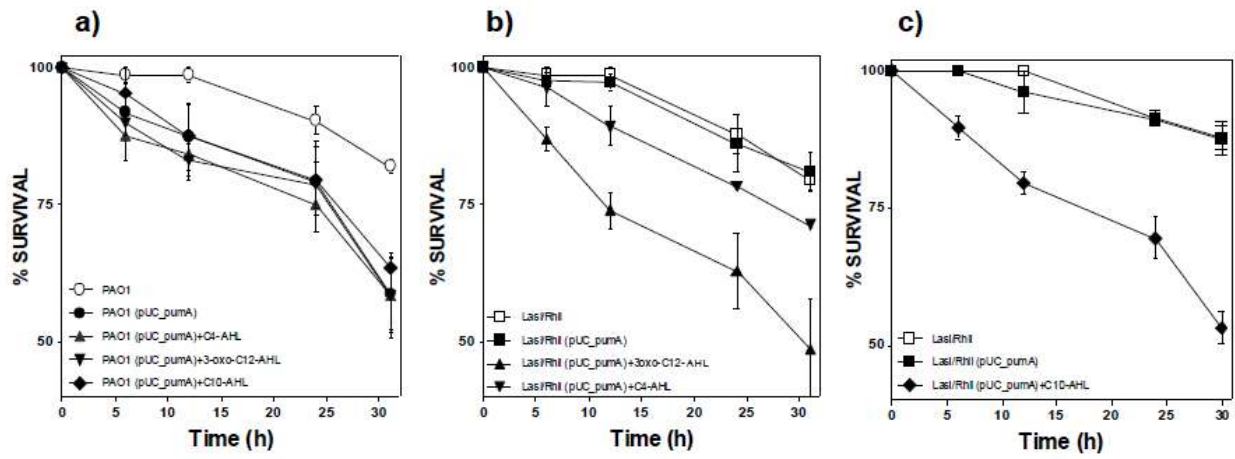


Figure 5.

IX. DISCUSIÓN GENERAL

Los sistemas Toxina-Antitoxina (TA) son elementos compuestos de dos genes, que están ampliamente distribuidos en los plásmidos y los cromosomas de las bacterias, que codifican a una toxina y una antitoxina respectivamente (Gerdes *et al.*, 2005; Harms *et al.*, 2016). Los sistemas TA se han relacionado con la estabilidad de plásmidos (sistemas TA codificados en plásmidos) y en la virulencia (sistemas TA codificados en cromosoma). En los sistemas TA, las toxinas son proteínas estables que inhiben el crecimiento o generan muerte celular, mientras que las antitoxinas regulan la actividad de la toxina, ya sea por su unión con la toxina o por la inhibición de la expresión del gen que la codifica (Leplae *et al.*, 2011; Page and Peti, 2016). Estos sistemas están vinculados a muchos aspectos de la fisiología celular, incluido el mantenimiento de plásmidos, la respuesta al estrés, la persistencia, la protección contra la infección por fagos y la formación de biopelículas (Wang and Wood, 2011; Otsuka, 2016). En aislados clínicos de *P. aeruginosa* con una alta capacidad para la generación de biopelículas se ha descrito una correlación en la presencia de genes de los sistemas TA y del sistema de quorum sensing (QS) (Hemati *et al.*, 2014). La expresión de los genes que codifican los sistemas TA está en equilibrio, mediante la regulación transcripcional de los operones. Generalmente en los sistemas TA tipo II, el complejo formado entre la toxina y la antitoxina reprime la transcripción de operones TA, porque las antitoxinas son capaces de unirse al DNA a través de un dominio de unión al DNA (Boss *et al.*, 2013). La autorregulación transcripcional se ha descrito para muchos sistemas TA de tipo II, generalmente a través del mecanismo de cooperatividad condicional, que es la regulación de la expresión de operones TA a través de la unión del complejo toxina-antitoxina,

complejo que debe mantener una la relación equimolar de la toxina y la antitoxina dentro de una célula (Page and Peti, 2016).

El sistema TA PumAB se identificó en el plásmido pUM505 de una cepa clínica de *P. aeruginosa*. Este sistema está formado por la toxina PumA y la antitoxina PumB. El sistema PumAB es codificado en un operón el cual permite la co-transcripción de los genes cuyos productos confieren estabilidad a plásmidos, al igual que otros sistemas TA codificados en plásmidos (Hernández-Ramírez, 2014). La transferencia de *pumA* a *P. aeruginosa* generó el aumento de la virulencia de la bacteria, además de mejorar su proliferación en tejidos de ratón, sugiriendo que la toxina PumA puede ser un factor de virulencia bacteriano. Por su parte, la proteína PumB, fue capaz de disminuir la proliferación de *P. aeruginosa* inducida por la proteína PumA, sugiriendo que PumB puede actuar como regulador de la actividad de PumA (Hernández-Ramírez, 2014). El sistema PumAB es un sistema TA no convencional, porque los genes del operón *pumAB* están organizados de manera diferente a la mayoría de los sistemas TA tipo II (Hernández-Ramírez, 2014). En los operones TA, generalmente el primer gen codifica una antitoxina, mientras que el segundo gen codifica a una toxina, y la regulación de su expresión es un rasgo característico de los complejos TA (Boss *et al.*, 2013). Además, las proteínas del sistema PumAB no son canónicas; PumA es similar a toxinas tipo RelE, mientras que PumB es similar a las antitoxinas tipo HTH (Hernández-Ramírez, 2014). Con base en las características del sistema PumAB, respecto a su organización genética y su papel en la virulencia bacteriana, el propósito de este trabajo fue estudiar el mecanismo de regulación de este sistema TA así como su control por el sistema de QS.

El análisis de la secuencia de la proteína PumA mostró baja identidad con proteínas tipo RelE ya que posee solo 22% de identidad con MjRelE de *Methanococcus jannaschii*, 18% con YoeB de *E. coli* y 14% con PhRelE de *Pyrococcus horikoshii* (Hernández-Ramírez, 2014), lo que no es sorprendente, ya que se ha reportado que las secuencias de aminoácidos de proteínas tipo RelE son bastante divergentes, incluso las que se originan de la misma especie bacteriana o estrechamente relacionadas (Goeders *et al.*, 2013). En general, las secuencias similares a RelE presentan de 24 al 47% de similitud entre sí y un 15 al 28% de identidad con la proteína RelE canónica de *E. coli* K-12 (Francuski and Saenger, 2009). Derivado de esto se procedió a realizar la predicción de la estructura secundaria de PumA y se comparó con las estructuras de otras proteínas de la familia RelE, mostrando que aunque es altamente divergente a el nivel de secuencia primaria, la proteína PumA contiene una estructura secundaria predicha que es característica de las toxinas de la superfamilia RelE, constituida por una lámina β de cinco hebras flanqueadas en un lado por α -hélices $\alpha 1$ y $\alpha 2$ y, por otro lado, por α -helices $\alpha 3$. Se ha informado que, a pesar de la baja conservación a nivel de secuencia de aminoácidos, las proteínas tipo RelE comparten estructuras secundarias y terciarias con plegamiento similar (Francuski and Saenger, 2009; Goeders *et al.*, 2013). Además, PumA contiene residuos conservados (Val4, Val41, Val44, Ile53, Val61 y Val81) con las proteínas MjRelE y YoeB de la superfamilia RelE. Este análisis sugirió que PumA podría pertenecer a la familia de toxinas RelE.

Por su parte, la predicción de la estructura secundaria de PumB mostró que la proteína contiene un dominio HTH de unión al DNA localizado en la región C-terminal de la proteína. El dominio HTH es característico de la familia Xre de

antitoxinas y este dominio a menudo se ha encontrado en antitoxinas caracterizadas (Yamaguchi *et al.*, 2011). En los sistemas TA de tipo II, la antitoxina es generalmente una proteína de unión al DNA que se une al operador del operón para reprimir su propia transcripción. En la mayoría de los casos, el motivo HTH se encuentra en el dominio N-terminal de las antitoxinas (Chan *et al.*, 2015). Ejemplos de la existencia del motivo HTH en estructuras resueltas de antitoxinas incluyen PezA (Khoo *et al.*, 2007), así como HigA (Schureck *et al.*, 2014). Este análisis sugiere que la proteína PumB podría funcionar como una proteína antitoxina de tipo HTH regulando la transcripción del sistema TA PumAB.

Como fue reportado por Hernández-Ramírez (2014) la sobreexpresión de PumA generó inhibición del crecimiento en *E. coli* BL21, por lo que en este trabajo se determinó si la inhibición por generada por PumA es neutralizada por su antitoxina cognado. Los resultados mostraron que la co-expresión de *pumA* y *pumB* no generó inhibición del crecimiento, sugiriendo que PumB se comporta como una proteína tipo antitoxina, ya que puede neutralizar los efectos inhibitorios del crecimiento generados por PumA. En los sistemas TA tipo II, la principal función de la antitoxina es neutralizar los efectos tóxicos de la toxina mediante su unión con la toxina (Yamaguchi *et al.*, 2011). Con el propósito de evaluar si PumA incrementa la virulencia de *P. aeruginosa* en otros modelos biológicos, el gen *pumA* fue transferido al nematodo *C. elegans*. Los resultados mostraron que al igual que en ratón, la transferencia de PumA incrementa la virulencia de *P. aeruginosa*. Además, se determinó que el sobrenadante libre de células (obtenido del cultivo de *P. aeruginosa* que contiene al gen *pumA*), es capaz de generar la muerte de los nematodos, sugiriendo que la proteína PumA es secretada por la bacteria. En

Rickettsia se ha descrito que el sistema TA vapBC TA genera efectos tóxicos dado a que la bacteria secreta a la toxina al citoplasma de las células del huésped (Audoly et al., 2011). Para determinar si la proteína PumA genera un efecto tóxico, se evaluó la muerte de los nematodos después de ser incubados con la proteína PumA pura, observando que PumA es capaz de matar a *C. elegans*, efecto que es drásticamente disminuido al desnaturalizar previamente a la proteína por medio de calor (95°C por 2 h) o por acción de una proteasa (Pronasa E). Esto indicó que los efectos tóxicos son generados por la proteína PumA, sugiriendo que la toxina requiere mantener una correcta estructura para su función. Finalmente, se evaluó la capacidad de la proteína PumB de neutralizar a PumA y así inhibir sus efectos tóxicos sobre *C. elegans*, para lo cual la proteína PumA fue previamente incubada *in vitro* con concentraciones variables de la proteína PumB y posteriormente adicionada a los nematodos. Se observó que cuando los nematodos fueron incubados con una mezcla de PumA y PumB en una proporción 1:2 (toxina:antitoxina), los efectos tóxicos generados por la proteína PumA disminuyeron, contrario a la toxicidad producida con la mezcla de toxina:antitoxina en una proporción 1:1, confirmando el papel de PumB en la neutralización y sugiriendo una relación molar entre PumA y PumB. La proporción toxina-antitoxina en los sistemas TA es importante para determinar la estequiometría del complejo TA así como regular la transcripción de los sistemas TA, fenómeno descrito como cooperatividad condicional. Este efecto además describe que el exceso de toxina puede perturbar la unión del operador por el complejo proteico TA (Hayes et al., 2014). Un ejemplo es el sistema CcdAB del plásmido F de *E. coli*, en donde ambos módulos forman un complejo (CcdA)₂-(CcdB)₂ que se une al DNA para regular el estado de represión del operón *ccd*, la

adición de mayor cantidad de CcdB al complejo proteína-DNA genera la eliminación de la represión del operón (Afif et al., 2001).

El análisis de los probables elementos reguladores ubicados en cis del operón *pumAB* mostró que, además de un probable promotor de tipo σ^{70} (P_{pumAB}), ubicado río arriba del operón *pumAB*, identificado previamente por Hernández-Ramírez (2014), este operón posee dos promotores adicionales, el promotor P_{pumB} (tipo σ^{70}) y el promotor $P_{pumB-AlgU}$ (tipo AlgU (σ^E)), ubicados río arriba del gen *pumB*. El factor σ^{70} de *P. aeruginosa*, llamado *rpoD*, un homólogo del factor σ^{70} de *E. coli*, reconoce una gran cantidad de promotores que controlan la expresión de genes de mantenimiento (Potvin et al., 2007). Mientras tanto, el factor σ_{AlgU} (también llamado RpoE o σ^{22}) comparte similitud (79% a nivel de aminoácidos) con la proteína de choque térmico *E. coli* σ^E , lo que sugiere funciones similares en respuesta al estrés (Potvin et al., 2008). La presencia de un promotor interno en operones de TA de sistemas de tipo II ya ha sido descrita. En *Enterococcus faecium*, el operón *ax-txe* posee un promotor principal río arriba del operón y un promotor interno que regula la expresión del gen *ax* (Boss et al., 2013). Más recientemente, de un estudio de 10 sistemas TA tipo II de *E. coli* K12 str. MG1655, se determinó que el operón *rnIAB* posee dos promotores y produce dos transcritos: un transcrito de todo el operón (RNAm de toxina y antitoxina) y un transcrito que solo puede traducir a la antitoxina (Deter et al., 2017). Por lo tanto, la presencia de múltiples promotores para el operón *pumAB* sugirió una regulación compleja de los genes.

El análisis de la expresión del operón *pumAB* reveló que los genes *pumA* y *pumB* se transcriben diferencialmente, siendo el RNAm de *pumB* más abundante que el transcrito de *pumA*. Esta diferencia en los niveles de RNAm de ambos genes fue

observada cuando se empleó RNA de cultivos de *E. coli* BL21 (pTrcHis_pumAB) en la fase de crecimiento exponencial tardía y en la fase de crecimiento estacionaria; cepa en donde la expresión del operón *pumAB* estaba bajo el control de un promotor inducido. Resultados similares fueron descritos para el operón *rnIAB* de *E. coli* K12 str. MG1655, que mostró una proporción diferente de RNAm de antitoxina respecto al de la toxina (Deter *et al.*, 2017). Sin embargo, los resultados mostrados por Deter *et al.* (2017) también mostraron que la mayoría de los sistemas TA producen un único transcrito, donde la concentración de RNAm de la antitoxina es aproximadamente igual a la concentración de la toxina, lo que sugiere que los sistemas TA aparentemente no están regulados diferencialmente a nivel transcripcional y que las diferentes tasas de traducción de las dos proteínas son responsables de la mayor producción de la proteína antitoxina que de la proteína de la toxina (Deter *et al.*, 2017).

Para determinar si la diferencia en los niveles de RNAm de los genes *pum* podría estar relacionada con la expresión individual de *pumB* por los promotores P_{pumB} y $P_{pumB-AlgU}$, se analizó la expresión de *pumB*, mostrand que *pumB* puede transcribirse sin *pumA*. El nivel de RNAm de *pumB* cambia con la fase de crecimiento, mostrando mayor transcripción durante la fase exponencial en comparación con la fase estacionaria, fortaleciendo la hipótesis de que la expresión de los genes *pum* puede regularse de manera diferencial por los promotores externos e internos del operón y por la etapa de crecimiento celular. En contraste con los operones TA previamente reportados, *axe-txe* y *rnIAB*, el operón *pumAB* posee dos promotores internos para la regulación de *pumB* (el tipo σ^{70} y el tipo AlgU (σ^E)), lo que sugiere la probable regulación diferencial del operón *pumAB*.

Se ha informado que, en condiciones normales de crecimiento, los sistemas TA permanecen inactivos y pueden activarse temporalmente en respuesta al estrés ambiental, así como al estrés nutricional y oxidativo (revisado por Hůrak and Tamman, 2017). El patrón de expresión de los genes de *pum* en condiciones estresantes fue similar al de las condiciones no estresantes durante la fase de crecimiento exponencial; sin embargo, los niveles de expresión del RNAm de *pumB* con respecto a *pumA* cambiaron durante la fase de crecimiento estacionaria, mostrando un 50% menos de la transcripción de *pumB*. Además, el estrés oxidativo solo afectó la expresión individual del gen *pumB* y no del gen *pumA*, sugiriendo que el promotor $P_{pumB-AlgU}$ controla la expresión de *pumB* en respuesta al estrés oxidativo durante la fase de crecimiento estacionaria. Se había informado previamente que en *P. aeruginosa*, AlgU está involucrado en la resistencia al estrés oxidativo y al choque térmico (Potvin *et al.*, 2008). Sin embargo, el factor sigma AlgU activa la transcripción de alginato (o mucoide) y su sobreproducción juega un papel importante en la patogénesis de esta bacteria al causar infección pulmonar en la fibrosis quística (Martin *et al.*, 1993). En esta bacteria, el regulón para la síntesis de flagelos está significativamente regulado por presencia de AlgU (Garrett *et al.*, 1999), lo que revela que la síntesis de alginato y la expresión del flagelo están inversamente reguladas por el factor sigma AlgU (Tart *et al.*, 2005; 2006). Esto sugiere que el promotor $P_{pumB-AlgU}$ está bajo el control negativo del factor sigma AlgU y que regula la transcripción de *pumB* bajo condiciones de estrés.

La proteína PumB posee un dominio HTH de unión al DNA, lo que sugiere que podría actuar como un regulador transcripcional de los genes en el operón *pumAB*. La unión de antitoxinas al DNA se relaciona con la autorregulación transcripcional

de sus propios operones; por lo tanto, la expresión y la activación de los sistemas TA están estrictamente reguladas por sus antitoxinas afines tanto a nivel transcripcional como postranscripcional (revisado en Schuster and Bertram, 2013). Mediante ensayos de movilidad electroforética (EMSA), se observó que la proteína PumB se une a la región de DNA donde se localiza el promotor P_{pumAB} del operón *pumAB*, sugiriendo que PumB regula la expresión del operón. El sitio probable donde se localiza la región operadora de operón está situada dentro de una secuencia palindrómica de 17 nt localizada entre el promotor P_{pumAB} y el codón de inicio de la secuencia codificante de *pumA*, ya que la eliminación del DNA correspondiente a esta secuencia palindrómica impide la unión de PumB con el DNA. PumB no pudo unirse a la región promotora de P_{pumB} y $P_{pumB-AlgU}$, ubicada en la región del extremo 5' del gen *pumB*, lo que sugiere que la regulación del operón *pumAB* es compleja, donde la transcripción del RNAm bicistrónico (que posee el RNAm de la toxina y antitoxina) está regulada por la antitoxina PumB y que la expresión individual del gen *pumB* está regulada por el factor AlgU.

En los sistemas TA tipo II, la autorregulación transcripcional de los operones TA generalmente es promovida por los complejos de antitoxinas o antitoxina-toxina que reprimen la transcripción a través de su unión a las secuencias palindrómicas en sus promotores (revisado por Otsuka, 2016). Además, PumA no pudo unirse al DNA de la región promotora/operadora P_{pumAB} del operón *pumAB*; sin embargo, la adición de PumA mejoró la unión de PumB, lo que sugiere la co-regulación de la expresión del operón *pumAB* por el complejo PumB-PumA. Por lo general, las antitoxinas de sistemas TA tipo II auto-reprimen su propio promotor, en forma individual o a manera de complejos con sus toxinas afines, y en algunos sistemas TA la regulación

transcripcional es regulada por cooperativa condicional que es dependiente de la estequiometría entre la toxina y la antitoxina (Schuster and Bertram, 2013). Como se indicó anteriormente, los genes *pumA* y *pumB* forman un operón y se co-transcriben como un RNAm bicistrónico (Hernández-Ramírez *et al.*, 2017); sin embargo, en este trabajo se observó que los niveles de RNAm de ambos genes son diferenciales. Para determinar que las diferencias en los niveles de transcrito entre ambos genes no se deben a la degradación del RNAm de *pumA*, se determinó el efecto de PumB sobre la estabilidad del transcrito de *pumA*. Inesperadamente, la adición *in vitro* de PumB purificado disminuyó específicamente los niveles del RNAm de *pumA* (cuando se expresaron individualmente) y no los otros RNAm analizados, indicando que la antitoxina afecta negativamente la estabilidad del RNAm de *pumA*. El efecto negativo de PumB sobre la estabilidad del RNAm de *pumA* se inhibió mediante la adición de inhibidores de la actividad de RNasa, lo que sugiere que PumB posee actividad de Endoribonucleasa sobre el RNAm de *pumA*. En general, la autorregulación transcripcional por la toxina y la antitoxina se ha descrito para muchos sistemas TA tipo II (Page and Peti, 2016); sin embargo, en el sistema TA tipo V, la antitoxina corta el RNAm de la toxina (Wang *et al.*, 2012). El sistema TA GhoT/GhoS fue el primer ejemplo de los sistemas TA tipo V. La antitoxina GhoS adopta un pliegue similar a la ferredoxina, similar a las endoribonucleasas específicas de la secuencia asociada a CRISPR-2 (CAS2), y corta el RNAm de *ghoT*, evitando su traducción en la toxina GhoT (Wang *et al.*, 2012). Nuestros resultados sugirieron que PumB posee una actividad RNasa similar a la antitoxina GhoS y tiene el potencial de escindir el RNAm de *pumA*. Sin embargo, en contraste con GhoS, PumB muestra la capacidad de unirse al DNA, lo que sugiere que

además regula la transcripción del operón *pumAB*, unión que es favorecida por la proteína PumA, lo que sugiere un mecanismo de cooperativa condicional.

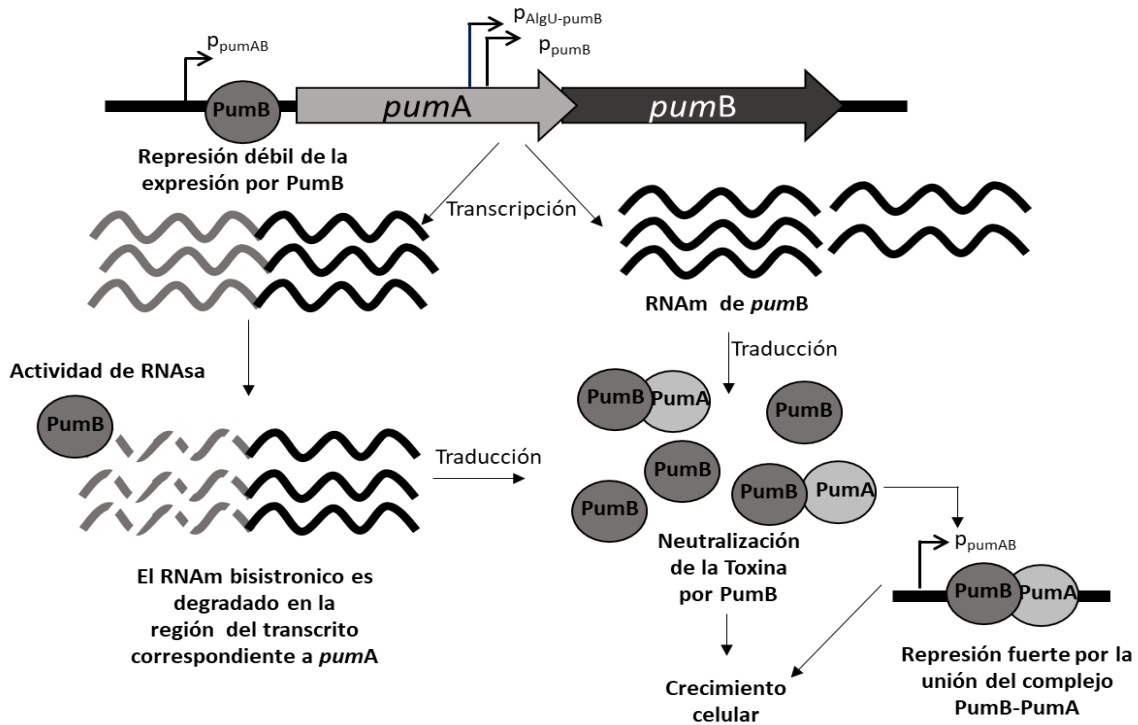
La transferencia de *pumA* a la cepa PAO1 de *P. aeruginosa* aumentó la virulencia bacteriana, sugiriendo que PumA puede ser un factor de virulencia (Hernández-Ramírez, 2014). En *P. aeruginosa* la producción de varios factores de virulencia (incluyendo la formación de biopelícula) es controlada por el sistema de QS (Hemati et al., 2014). Además, algunos sistemas TA tienen un papel importante en la formación de biopelículas, reportándose una correlación entre la capacidad de producción de biopelículas y la presencia de genes de QS y sistemas TA (Wang et al., 2011; Wen et al., 2014; Kedzierska and Hayes, 2016). La transferencia de *pumA* en la doble mutante de *P. aeruginosa* lasI/rhlI (mutante en los sistemas LasI y RhII de QS) no generó aumento en la virulencia de la bacteria en lechuga y en *C. elegans*; contrario a lo observado con la transferencia de gen *pumA* en *P. aeruginosa* PAO1. Además, los niveles de RNAm de *pumA* disminuyeron drásticamente en el fondo mutante *P. aeruginosa* lasI/rhlI, lo que sugiere que la expresión de *pumA* es regulada por QS e indica que la virulencia conferida por PumA es dependiente de QS. Se ha demostrado que QS controla la expresión de genes de virulencia en bacterias Gram negativas y Gram positivas, fenómeno ha demostrado ser relevante para la virulencia bacteriana *in vivo* (Antunes et al., 2010). Nuestros datos sugieren que la toxina PumA del sistema TA PumAB codificado en plásmido, actúa como un factor de virulencia que podría ser regulado por QS, fortaleciendo la hipótesis de una correlación entre los sistemas QS y TA.

Varios sistemas QS que se encuentran en muchos patógenos bacterianos producen y liberan pequeñas señales químicas llamadas autoinductores que comúnmente se

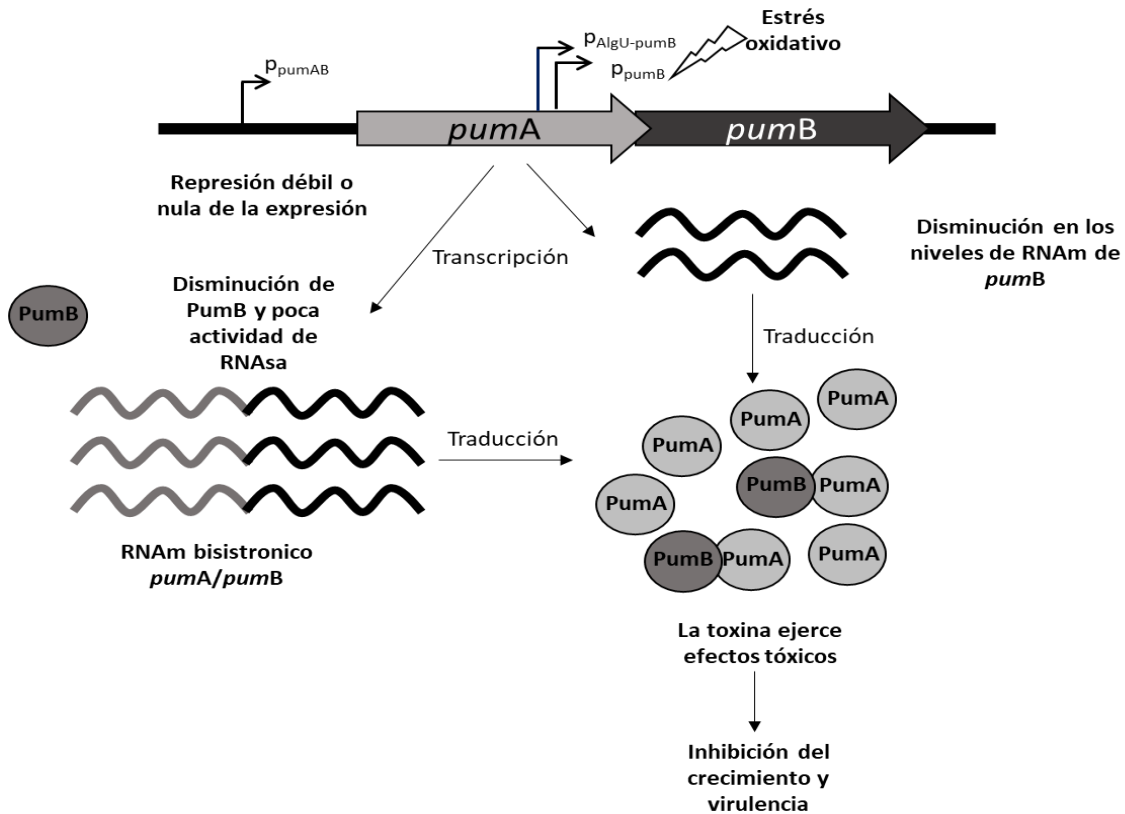
asocian con la regulación de genes que codifican la producción de factores de virulencia y la formación de biopelículas (Whitehead *et al.*, 2001; Ng and Bassler, 2009; Deng *et al.*, 2011; Pereira *et al.*, 2013). En *P. aeruginosa* el gen *lasI* sintetiza 3-oxo-C12-AHL, una molécula de acil homoserina lactona (autoinductor), y el gen *rhII* sintetiza C4-AHL, un segundo autoinductor (Pearson *et al.*, 1994). La complementación química por la adición del 3-oxo-C12-AHL y C4-AHL a cultivos de *P. aeruginosa lasI/rhII* (pUC_pumA) incrementó los niveles de RNAm de *pumA* y restauró la virulencia de la bacteria en *C. elegans*, indicando que ambos sistemas LasI y RhII del QS regulan la función de PumA.

Con los resultados obtenidos en este trabajo proponemos un modelo que sugiere el mecanismo de regulación del sistema PumAB a través de la función de PumB, por el estrés oxidativo, así como por parte del sistema de QS (**Figura 7**).

A) Regulación del sistema TA PumAB durante el crecimiento en ausencia de estrés



B) Regulación del sistema TA PumAB durante el crecimiento en presencia de estrés



C) Regulación de la expresión de PumA del sistema TA PumAB por QS

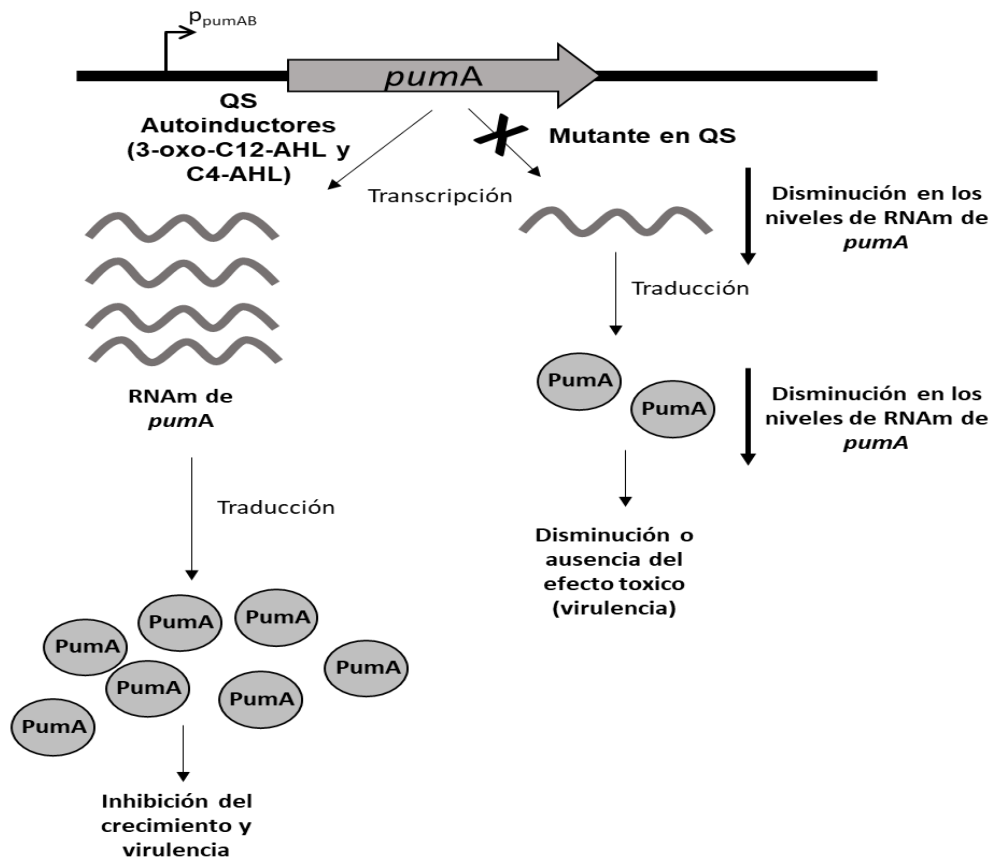


Figura 7. Descripción del modelo de la regulación transcripcional del operón *pumAB*. **A)** Control de la expresión de los genes *pumAB* durante el crecimiento en ausencia de estrés: los genes *pumA* y *pumB* son co-transcritos por el promotor P_{pumAB} , mientras que el gen *pumB* es transcrito individualmente por los promotores P_{pumB} y $P_{AlgU-pumB}$. En esta condición, se favorece la transcripción de *pumB*, lo que sugiere niveles más altos de PumB sintetizados con respecto a la proteína PumA. La proteína PumB podría unirse sola (represión débil) o en complejo con la toxina PumA (represión fuerte) al operador del operón *pumAB* autorregulando la co-expresión de los genes *pumAB*. PumB podría cortar el RNAm de *pumA* evitando su transducción, aunado a que PumB podría formar un complejo proteína-proteína con PumA neutralizando la toxina. Finalmente, bajo esta condición, se podría favorecer el crecimiento celular. **B)** Control de la expresión de genes *pumAB* en condiciones

de crecimiento en presencia de estrés oxidativo: los genes *pumA* y *pumB* son co-transcritos por el promotor p_{pumAB} , mientras que la expresión individual del gen *pumB* es regulada negativamente en los promotores p_{pumB} y/o $p_{AlgU-pumB}$ disminuyendo el RNAm de *pumB*. Bajo esta condición, se favorece de la transcripción *pumA*. Además, como ha sido descrito para otras antitoxinas, la antitoxina de PumB producida podría ser degradada por el estrés oxidativo provocando una disminución en la concentración de PumB. Esto sugiere que en condiciones de estrés oxidativo los niveles de antitoxina PumB no son suficientes para la represión del operón *pumAB*, para neutralizar a la proteína PumA, así como para degradar el RNAm de *pumA*, por lo que la toxina podría actuar contra sus objetivos e inhibir el crecimiento celular.

C) Control de la expresión de los genes *pumAB* por QS: al producirse los autoinductores 3-oxo-C12-AHL y C4-AHL del QS, los genes *pumA* y *pumB* son co-transcritos, produciéndose la toxina y la antitoxina, en esta condición la expresión individual de *pumA* ejerce efectos tóxicos al hospedero de la bacteria. En un fondo mutante en QS la bacteria no produce los autoinductores, los niveles de expresión de *pumA* y la coexpresión de *pumA* y *pumB* disminuyen. Durante esta condición la expresión individual de *pumA* disminuye, sugiriendo la disminución en la concentración de la toxina PumA y por consiguiente una disminución o ausencia de virulencia.

X. CONCLUSIÓN

La regulación del sistema PumAB del plásmido pUM505 ocurre por múltiples mecanismos como son: 1) la neutralización de la función toxica de la toxina PumA por la proteína PumB, probablemente mediante la interacción proteína-proteína; 2) la regulación a nivel transcripcional por PumB o el complejo PumB-PumA, por estrés oxidativo, así como por el sistema de QS y 3) por la actividad de ribonucleasa de PumB sobre el RNAm de *pumA*. La compleja regulación del sistema PumAB podría estar relacionada con la función del sistema PumAB en la estabilidad de plásmidos y la virulencia.

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XII. ADENDA

- 1.- Chavez-Jacobo, V., **Hernández-Ramírez, K. C.**, Romo-Rodríguez, P., Pérez-Gallardo, R. V., Campos-García, J., Guitiérrez-Corona, J. F., García-Merinos, J. P., Meza-Carmen, V. y Ramirez-Díaz, M. I. (2018) Novel Ciprofloxacin-Modifying Enzyme (CrpP) Encoded by the *Pseudomonas aeruginosa* pUM505 Plasmid. *Antimicrob Agents Chemother.* 62(6):AAC.02629-17.
- 2.- **Hernández-Ramírez, K. C.**, Reyes-Gallegos, R. I., Chavez-Jacobo, V., Díaz-Magaña, A., Meza-Carmen, V. y Ramirez-Díaz, M. I. (2018) A plasmid-encoded mobile genetic element from *Pseudomonas aeruginosa* that confers heavy metal resistance and virulence. *Plasmid.* 98.
- 3.- Chavez-Jacobo, V., **Hernández-Ramírez, K. C.**, Cervantes, C., Meza-Carmen, V. y Ramirez-Díaz, M. I. (2019) Prevalence of the *crpP* gene conferring decreased ciprofloxacin susceptibility in enterobacterial clinical isolates from Mexican hospitals. *J Antimicrob Chemother.* 74:5.
- 4.- Artículo de divulgación: **Hernández-Ramírez K. C.** Plásmidos bacterianos. Revista de divulgación científica: Saber Más. 2017. No. 36. http://www.sabermas.umich.mx/revistas/No_33.pdf.



CrpP Is a Novel Ciprofloxacin-Modifying Enzyme Encoded by the *Pseudomonas aeruginosa* pUM505 Plasmid

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ABSTRACT The pUM505 plasmid, isolated from a clinical *Pseudomonas aeruginosa* isolate, confers resistance to ciprofloxacin (CIP) when transferred into the standard *P. aeruginosa* strain PAO1. CIP is an antibiotic of the quinolone family that is used to treat *P. aeruginosa* infections. *In silico* analysis, performed to identify CIP resistance genes, revealed that the 65-amino-acid product encoded by the *orf131* gene in pUM505 displays 40% amino acid identity to the *Mycobacterium smegmatis* aminoglycoside phosphotransferase (an enzyme that phosphorylates and inactivates aminoglycoside antibiotics). We cloned *orf131* (renamed *crpP*, for ciprofloxacin resistance protein, plasmid encoded) into the pUCP20 shuttle vector. The resulting recombinant plasmid, pUC-*crpP*, conferred resistance to CIP on *Escherichia coli* strain J53-3, suggesting that this gene encodes a protein involved in CIP resistance. Using coupled enzymatic analysis, we determined that the activity of CrpP on CIP is ATP dependent, while little activity against norfloxacin was detected, suggesting that CIP may undergo phosphorylation. Using a recombinant His-tagged CrpP protein and liquid chromatography–tandem mass spectrometry, we also showed that CIP was phosphorylated prior to its degradation. Thus, our findings demonstrate that CrpP, encoded on the pUM505 plasmid, represents a new mechanism of CIP resistance in *P. aeruginosa*, which involves phosphorylation of the antibiotic.

KEYWORDS *Pseudomonas aeruginosa*, ciprofloxacin, enzyme, phosphorylation, plasmid-mediated resistance, quinolones

Quinolones are synthetic antibiotics that constitute an important class of biologically active, broad-spectrum antibacterial drugs (1). These antibiotics are used in humans to treat a variety of bacterial infections and represent one of the most commonly prescribed classes of antibacterial drugs in the world (2). Ciprofloxacin (CIP), a fluoroquinolone, was the first quinolone to display considerable activity outside the urinary tract (3). Due to the pharmacological characteristics of CIP, as well as its potency, activity spectrum, oral bioavailability, and good safety profile, this antibiotic has been extensively used clinically worldwide (4). Importantly, CIP is the most active quinolone against *Pseudomonas aeruginosa* and is used to treat infections generated by this bacterium. CIP is also used to treat infections with various Gram-negative and Gram-positive bacteria and is the most commonly prescribed antibacterial drug to date according to guidelines (4, 5).

The mechanism of action of quinolone antibiotics consists of the inhibition of DNA gyrase and topoisomerase IV (Topo IV), enzymes that modulate the topological state of

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TABLE 1 Results of susceptibility tests

Antibiotic	MIC ($\mu\text{g/ml}$) for:						
	<i>P. aeruginosa</i>				<i>E. coli</i>		
	ATCC 27853	PAO1(pUCP20)	PAO1(pUC_crpP)	PAO1(pUM505)	ATCC 25922	J53-3(pUCP20)	J53-3(pUC_crpP)
Ciprofloxacin	0.5	0.5	0.5	2.0	0.004	0.008	0.06
Levofloxacin	0.5	1.0	1.0	1.0	0.008	0.008	0.008
Norfloxacin	0.5	0.5	0.5	2.0	0.03	0.03	0.03
Moxifloxacin	1.0	2.0	2.0	8.0	0.008	0.03	0.03
Nalidixic acid	>256	>256	>256	>256	2.0	8.0	8.0
Kanamycin	>256	32	32	32	4.0	4.0	2.0
Streptomycin	8.0	8.0	4.0	4.0	8.0	4.0	4.0
Amikacin	1.0	0.5	0.5	0.5	2.0	1.0	1.0
Gentamicin	0.5	0.5	0.5	0.5	1.0	0.5	0.5

the DNA and are essential for transcription, DNA replication, and the repair/recombination of DNA (1). These two enzymes display structural and functional similarities; however, their specific mechanisms of action during DNA replication differ (6). The quinolones stabilize the cleavage complexes by binding the enzyme-DNA interface in the cleavage-ligation active site, resulting in the inhibition of DNA ligation (1). Therefore, the intercalation of quinolones into DNA results in increased cell death (1).

Resistance to quinolones can involve chromosomal gene mutations or may be plasmid mediated. A correlation between CIP resistance and the number of resistance-associated alterations in GyrA, GyrB subunits of topoisomerase II, and ParC and ParE subunits of topoisomerase IV in *P. aeruginosa* isolates has been reported previously (7). In *Escherichia coli*, mutations in *marR* (a *marA* repressor) lead to the MarA-mediated activation of genes encoding the AcrAB-TolC efflux pump, which plays a major role in quinolone efflux. The *marR* mutations also decrease the translation of *ompF*, a gene encoding the OmpF outer membrane porin, thus decreasing quinolone influx (4). Plasmid-mediated quinolone resistance (PMQR) confers decreased susceptibility to these antibiotics. Some examples of PMQR systems include the Qnr protein, which prevents quinolone binding to target proteins, and QepA, an efflux pump that decreases susceptibility to CIP and norfloxacin (NOR) (4, 6). Moreover, mutations in the gene encoding the aminoglycoside *N*-6'-acetyltransferase [AAC(6')-Ib] enzyme, which confers resistance to aminoglycoside antibiotics, results in a variant [AAC(6')-Ib-cr] that promotes CIP and NOR resistance (8).

pUM505 is a self-conjugating 123-kbp plasmid isolated from a clinical *Pseudomonas aeruginosa* isolate (9). This plasmid carries several adaptive genes, including the *umuD* gene (encoding a transcriptional regulator of the SOS response) (10), genes involved in *Pseudomonas* virulence (11, 12), and genes that increase plasmid stability (12). The aim of this work was to identify and study the product of the *orf131* (renamed *crpP*) gene on pUM505, which confers resistance to CIP. We show that the mechanism underlying this resistance to CIP involves CIP phosphorylation.

RESULTS

Quinolone resistance is conferred by the pUM505 plasmid. The pUM505 plasmid, originally isolated from a clinical isolate of *P. aeruginosa* (9), confers resistance to CIP when transferred into *P. aeruginosa* strain PU21. This observation suggested that pUM505 encodes a factor involved in susceptibility to this antibiotic (13). To determine whether pUM505 confers resistance to other quinolones, the pUM505 plasmid was transferred into *P. aeruginosa* strain PAO1, and the antimicrobial susceptibilities to CIP, NOR, levofloxacin (LVX), moxifloxacin (MXF), and nalidixic acid (NAL) were investigated. Our results showed that CIP, NOR, and MXF displayed 4-fold higher MICs for *P. aeruginosa* PAO1(pUM505) than for the *P. aeruginosa* reference strain PAO1. In addition, the MIC values for strain PAO1 were similar to those measured for the *P. aeruginosa* reference strain, ATCC 27853 (Table 1). These findings indicated that the pUM505 plasmid encodes a protein involved in quinolone resistance.

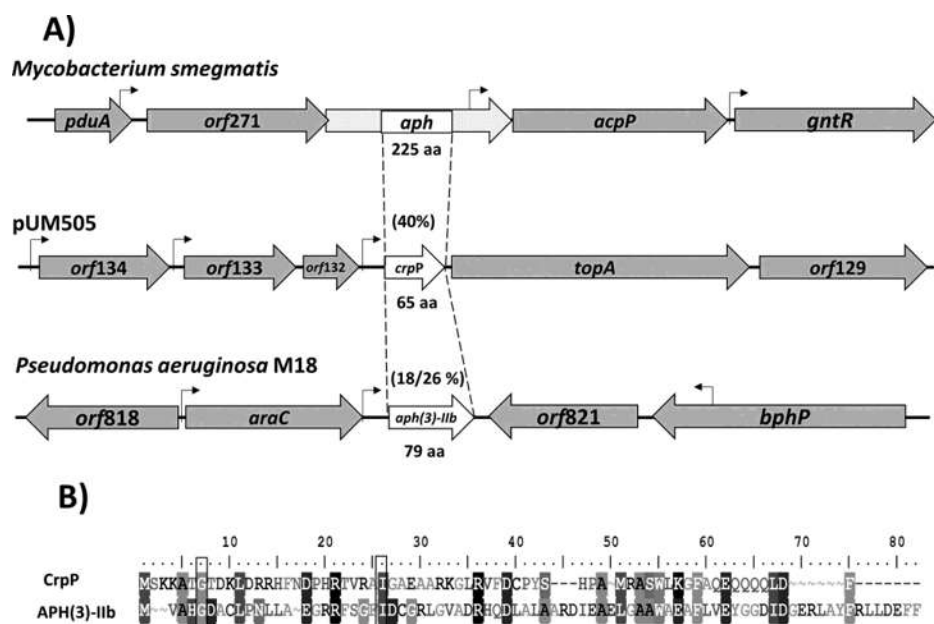


FIG 1 Comparison of CrpP to aminoglycoside phosphotransferases. (A) The coding regions and transcriptional directions of the *aph*, *crpP*, and *aph(3)-IIB* genes from *M. smegmatis*, the pUM505 plasmid, and *P. aeruginosa* M18, respectively, are indicated by open arrows, with the sizes of predicted polypeptides (in amino acids) indicated. Percentages of amino acid identity/similarity for the proteins compared are given in parentheses; stippled areas of the *aph* gene from *M. smegmatis* were not considered for the alignments. Shaded arrows represent adjacent genes, and the locations of the putative promoters' sequences are marked by bent arrows. (B) Alignment of the putative protein encoded by the *crpP* gene with the APH(3)-IIB protein from *P. aeruginosa* M18. Asterisks denote conserved catalytic residues in the APH(3)-IIB gene from *P. aeruginosa* M18. Filled squares indicate conserved catalytic residues identified in the protein encoded by the *crpP* gene.

Identification of the *crpP* gene. *In silico* analysis of pUM505 failed to identify a PMQR element (13). Moreover, a BLAST analysis revealed that the protein encoded by the *orf131* gene (RefSeq accession number [NC_016138](#); region, nucleotides 119072 to 119269; renamed *crpP*, for ciprofloxacin resistance protein, plasmid encoded) shares 40% identity with a 42-amino-acid (42-aa) region of the aminoglycoside phosphotransferase (APH) from *Mycobacterium smegmatis* (Fig. 1A). The *crpP* gene possesses a putative sigma-70 promoter in the 5' region and is predicted to encode a 65-aa protein (Fig. 1A; see also Fig. S1A in the supplemental material). In contrast, the APH protein from *M. smegmatis* contains 225 aa and is much longer than the protein encoded by *crpP*; however, several members of this protein family are smaller. For example, the 79-aa aminoglycoside phosphotransferase [APH(3)-IIB] from *P. aeruginosa* M18 confers kanamycin (KAN), gentamicin (GEN), and streptomycin (STR) resistance (14). CrpP and APH(3)-IIB display a low degree of identity (18%) and 26% similarity (Fig. 1A); however, CrpP contains two residues conserved in APH enzymes, 7-Gly (involved in catalysis) and 26-Ile (involved in ATP binding) (Fig. 1B). Hence, these analyses suggest the product encoded by the *crpP* gene may confer resistance to antibiotics.

The protein encoded by *crpP* confers resistance to CIP. To test whether *crpP*, including the 5' putative regulatory region, is involved in resistance to CIP, the regions were amplified by PCR and were cloned into the high-copy-number vector pUCP20. The pUC-*crpP* recombinant plasmid was transferred into *P. aeruginosa* strain PAO1, and susceptibility to CIP was determined. Our results showed that *P. aeruginosa* strain PAO1 expressing the pUC-*crpP* plasmid had a level of susceptibility to CIP similar to that observed with strain PAO1 (Table 1). These results suggest that the *crpP* product does

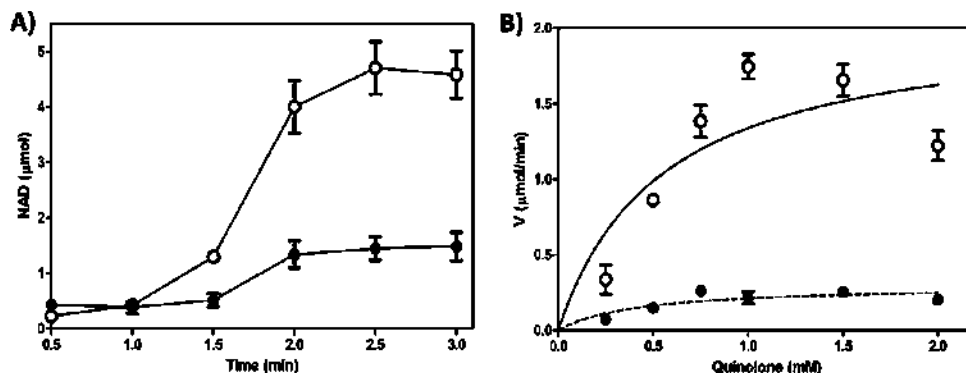


FIG 2 CrpP enzyme kinetics. (A) NADH oxidation rate comparisons for CIP (○) and NOR (●); 2.0 mM each antibiotic was incubated in the presence of 5.0 $\mu\text{g/ml}$ His-CrpP protein at 37°C for 15 min. NADH oxidation was measured spectrophotometrically at 390 nm. (B) Michaelis-Menten plots for CIP (○) and NOR (●). The plot shows the NADH oxidation data. Data are means from four independent duplicate assays, with standard errors.

not confer significant resistance to quinolones in the genetic background of *P. aeruginosa* PAO1, a strain known for its intrinsic high resistance to many antibiotics (15). However, another explanation may be that in PAO1, the *crpP* gene is not expressed under the control of its own promoter in the pUC20 vector. To distinguish between these possibilities, *crpP* gene expression in cultures of *P. aeruginosa* PAO1 containing the recombinant pUC-*crpP* plasmid was determined by quantitative reverse transcription-PCR (RT-qPCR) assays. The results showed that the *crpP* gene was expressed in *P. aeruginosa* strain PAO1 (Fig. S1B in the supplemental material). This finding suggested that this gene is functional in this strain. To determine whether *crpP* confers susceptibility to quinolones, the more-susceptible *E. coli* J53-3 bacterial background was used. MIC assays with *E. coli* J53-3(pUC-*crpP*) showed a 7.5-fold decrease in susceptibility to CIP, while susceptibilities to the other four quinolones tested (LVX, NOR, MXF, and NAL) did not differ from those of *E. coli* strain J53-3 (Table 1). Additionally, RT-qPCR analysis of expression showed that the *crpP* gene was expressed in this strain (Fig. S1B). These results indicated that *crpP* encodes a protein that confers decreased susceptibility to CIP on *E. coli*. In addition, the susceptibilities of *E. coli* J53-3(pUC-*crpP*) to KAN, STR, amikacin (AMK), and GEN were similar to those of the *E. coli* control strain (Table 1), suggesting that the *crpP* gene does not confer resistance to these aminoglycosides. These results indicated that the *crpP* gene on the pUM505 plasmid encodes a protein that confers selective resistance to CIP alone.

Enzymatic activity of the CrpP protein. Because our sequence analysis suggested that *crpP* encodes a protein that is similar to APH antibiotic-modifying enzymes, we next determined the *in vitro* activity of the purified CrpP enzyme. To purify CrpP, the coding region of *crpP* was cloned into an overexpression vector, and a recombinant His-tagged CrpP protein was isolated, as described in Materials and Methods. We first tested whether the recombinant His-CrpP protein was active in cells *in vivo*. To this end, we used isopropyl β -D-1-thiogalactopyranoside (IPTG) to induce overexpression of the His-CrpP protein in *E. coli* BL21(pTrcHis-*crpP*) grown in the presence or absence of CIP. Upon His-CrpP induction, this strain grew better than the noninduced strain in the presence of CIP (see Fig. S2 in the supplemental material). This finding suggested that the N-terminal 6 $\times$ His tag did not alter the activity of the enzyme, and therefore the His tag was retained as part of the recombinant protein for all subsequent experiments.

To determine the activity of CrpP on CIP, we used a coupled enzymatic assay involving NADH oxidation (16). In the presence of CrpP, NADH oxidation increases proportionally with the reaction time (Fig. 2A). Moreover, NADH oxidation also increased proportionally with the amount of protein in the assay (see Fig. S3 in the

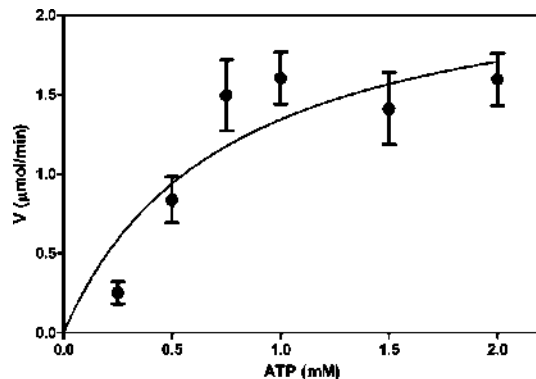


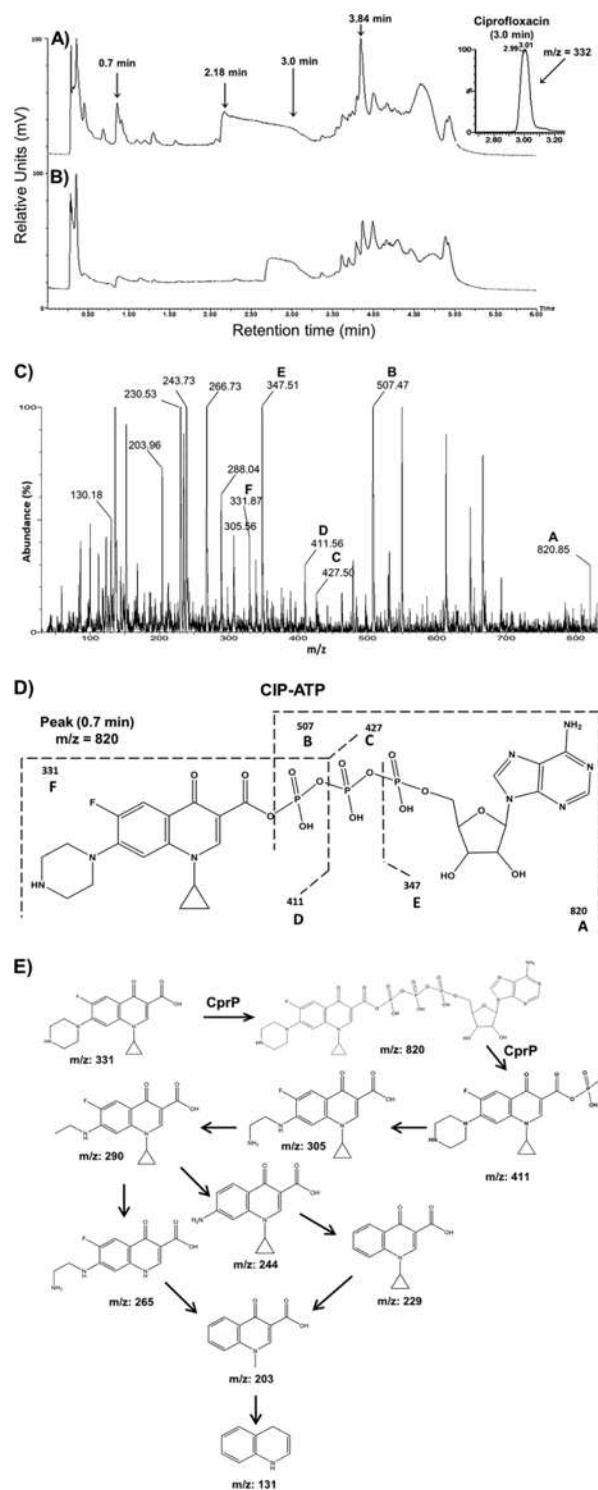
FIG 3 Michaelis-Menten plot of His-CrpP activity on ATP. ATP was incubated at the indicated concentrations in the presence of 5 μ g/ml His-CrpP protein at 37°C for 15 min, as described in Materials and Methods. The Michaelis-Menten plot shows the data for NADH oxidation, which was measured spectrophotometrically at 390 nm. Data are means and standard errors from four independent duplicate assays.

supplemental material), indicating that CIP is a substrate of CrpP. Additionally, we determined the activity of CrpP on CIP following heat-induced CrpP denaturation. This resulted in a reduced level of CrpP activity from that of the native protein at the same concentration (Fig. S3), suggesting that the activity was specific to CrpP and that the correct structure of the protein is required for its function. Additionally, we analyzed the effects of pH and Mg^{2+} concentration on CrpP activity. The optimal pH was 7.0, and optimal CrpP activity was observed when 10 mM $MgCl_2$ was used (see Fig. S4 in the supplemental material).

The MICs indicated that *crpP* confers resistance to CIP only, suggesting an enzymatic activity that is specific to this antibiotic; thus, we analyzed the catalytic activity of CrpP against NOR, LVX, MXF, and NAL. We observed a lower level of CrpP activity when NOR was used as a substrate than when CIP was used (Fig. 2A), and no activity against LVX, MXF, or NAL was observed (see Fig. S5 in the supplemental material). These results are consistent with the susceptibility to quinolones observed by measuring MICs (Table 1) and suggest that the low level of CrpP activity on NOR does not enable detection of a decreased susceptibility to this antibiotic in *E. coli*. Moreover, CrpP cannot use aminoglycosides, such as KAN and STR, as substrates, suggesting that CrpP is specific for CIP and NOR (Fig. S5).

We also estimated the apparent kinetic constants of CrpP for CIP and NOR (Fig. 2B). We detected a K_m of 0.5483 ± 0.2762 mM and a V_{max} of 2.071 ± 0.3745 μ mol/min for CIP and a K_m of 0.4192 ± 0.2208 mM and a V_{max} of 0.2989 ± 0.4965 μ mol/min for NOR. The V_{max}/K_m ratio was approximately 5-fold higher for CIP than for NOR (3.77 for CIP versus 0.713 for NOR). These *in vitro* results demonstrated that CrpP is less efficient at utilizing NOR than it is at utilizing CIP as a substrate. In addition, when ATP was used as the variable cosubstrate, CrpP activity changed as a function of the ATP concentration (Fig. 3), indicating that the activity of CrpP was ATP dependent. This further suggested that the mechanism underlying CrpP function involves phosphorylation, considering that APHs catalyze the transfer of a phosphate group to the aminoglycoside molecule, and several of these enzymes use ATP as the phosphate donor group (17). Taken together, these results indicated that CrpP confers a selective resistance to CIP, probably through a mechanism involving phosphorylation.

To elucidate the mechanism of resistance to CIP, metabolite analysis was performed *in vitro* using liquid chromatography–tandem mass spectrometry (LC–MS–MS). When CIP was incubated with the His-CrpP protein, three peaks of interest, with retention times of 0.7, 2.18, and 3.84 min, were observed. These peaks were not observed in the chromatogram of the reaction in the absence of His-CrpP or ATP (Fig. 4A and B), suggesting a modification of the CIP molecule. Analysis of the peak with a retention



time of 0.7 min clearly indicated the presence of a molecular ion corresponding to CIP-ATP (m/z 820) (Fig. 4C). In addition, several intermediary molecular ions were also identified, such as those with m/z 507, m/z 427, m/z 411, m/z 347, and m/z 331; the last ion corresponds to the CIP molecule (Fig. 4D). To better understand the mechanism of CIP inactivation, we analyzed the peaks showing retention times of 2.18 and 3.84 min (Fig. 4A). We detected mass ions corresponding to metabolites produced following fragmentation of the molecule. We propose that CIP is first activated by the binding of the ATP molecule (m/z 820), followed by phosphorylation of the molecule, confirmed by the presence of CIP monophosphate (m/z 411) (Fig. 4E). In addition, infrared spectroscopy assays showed that His-CrpP induced changes in the intensities of the characteristic absorption bands of the carboxyl group in the 2,500-to-3,300 cm^{-1} , 1,200-to-1,420 cm^{-1} , and 1,781 cm^{-1} regions, as well as in the 1,675 cm^{-1} absorption band, which corresponds to the vibration of the ketone carbonyl group at C-3 of the CIP molecule (see Fig. S6 in the supplemental material). This result agrees with the modification of CIP determined by LC-MS-MS, suggesting that CIP is phosphorylated on the carboxyl group. Additionally, these results suggest that the entire structure of CIP is important for CrpP function, since the carboxyl group is part of the central ring system in the quinolones, but CrpP did not show activity against other quinolones. A probable explanation for these results is that LVX and MXF contain oxygenated groups at position 8 of the central ring system in the quinolones, which can provide activation on the aromatic moiety, resulting in a higher electronic density in this part of the molecule. Meanwhile, the ethyl group of position 1 of the central ring of the quinolones in NOR could inject less electron density on the nitrogen atom than the cyclopropyl group present in CIP, producing, as a result, greater reactivity on the carboxyl group in CIP than in the other quinolones. In addition, metabolite analysis when CIP was incubated with crude extracts of an *E. coli* strain overexpressing the His-CrpP protein and ATP showed peaks corresponding to several degradation intermediaries of the phosphorylated CIP (CIP-P) molecule. These included molecular ions such as those with m/z 305, m/z 290, m/z 265, m/z 244, m/z 229, m/z 203, and m/z 131 (Fig. 4E; see also Fig. S7 in the supplemental material), suggesting that after CIP phosphorylation, the molecule is degraded.

DISCUSSION

The pUM505 plasmid is a conjugative plasmid isolated from a clinical isolate of *P. aeruginosa* (9). pUM505 carries different genes, which encode proteins involved in metal resistance, DNA repair, virulence, and plasmid stability (10–13). Additionally, pUM505 conferred CIP resistance on *P. aeruginosa* PU21 (13), suggesting that pUM505 contains genes responsible for quinolone resistance. Because *in silico* analysis of the pUM505 genes did not initially identify genes previously implicated in quinolone resistance (13), these data suggested that the CIP resistance transferred by pUM505 could be conferred by genes not previously reported. The bacterial resistance to quinolones reported to date may involve chromosomal gene mutations or may be plasmid mediated (4, 6). In addition, these systems could provide resistance against several quinolones. For example, the QepA efflux pump decreases susceptibility to CIP and NOR (4, 6). For this reason, we determined whether pUM505 could confer resistance to other quinolones. The results showed that pUM505 confers decreased susceptibility to the fluoroquinolones CIP, NOR, and MXF in *P. aeruginosa* strain PAO1. To

FIG 4 LC-MS-MS analysis of metabolites following CIP inactivation. (A and B) Representative chromatograms correspond to reaction mixtures containing the His-CrpP protein in the presence of CIP and ATP (A) or in the absence of ATP (B). The reactions were conducted as described in Materials and Methods. Arrows indicate peaks showing CIP structures (retention times of 0.7 min, 2.18 min, 3.0 min, and 3.84 min). (C) Representative mass fragmentation profile corresponding to the peak with a 0.7-min retention time. Capital letters above the peaks indicate the molecular ions that correspond to fragmented molecules derived from the ciprofloxacin-ATP molecule. Peak A (m/z 820) corresponds to the CrpP-ATP molecular ion. (D) Structure analysis of the mass fragmentation profile from panel C. (E) Reaction mechanism of CIP inactivation/fragmentation by the His-CrpP protein. The structures and m/z ratios were subtracted from the mass fragmentation profiles of peaks with retention times of 0.7, 2.18, and 3.84 min shown in panel A. Mass fragmentation profiles are shown in Fig. S7 in the supplemental material.

elucidate the molecular elements involved in fluoroquinolone resistance by pUM505, a new *in silico* analysis of the genes carried by this plasmid was conducted. We identified the 65-aa protein encoded by the *crpP* gene as a unique protein that shares sequence similarity with other proteins involved in resistance to antibiotics. CrpP displayed low identity (18%) to the APH-IIb enzyme from *P. aeruginosa* M18. The APH-IIb enzyme confers KAN, GEN, and STR resistance by transferring the phosphoryl group of ATP to the 3-hydroxyl groups of these antibiotics (14, 18). However, while APH enzymes share low (20 to 40%) sequence identity (18), they contain important conserved motifs, particularly at the C terminus, which is primarily responsible for catalysis and aminoglycoside binding. In addition, the ATP-binding domain is mainly located at the N terminus (18). The CrpP protein does not possess the catalytic motifs reported for APH enzymes, although it contains two conserved catalytic residues, 7-Gly (involved in catalysis) and 26-Ile (involved in ATP binding). In addition, it has been reported that the sequences of the catalytic and ATP-binding domains differ between individual APH enzymes and between APH subfamilies (18). Thus, the CrpP protein may confer resistance to antibiotics, while variability in the protein sequence (relative to those of APH enzymes) may promote the recognition of specific substrates.

The presence of the *crpP* gene did not decrease the quinolone susceptibility of *P. aeruginosa* strain PAO1, possibly because this strain has intrinsic high resistance to many antibiotics (15). However, the transfer of pUM505 to *P. aeruginosa* PAO1 was able to confer decreased susceptibility to CIP, NOR, and MXF. These results suggested that pUM505 might encode, in addition to the *crpP* gene, several mechanisms of resistance that, together, could increase *Pseudomonas* resistance to quinolones. It has been reported that PMQR elements [Qnr proteins, the AAC(6')-Ib-cr enzyme, or the OqxAB, QepA1, and QepA2 efflux pump proteins] could confer reduced susceptibility to quinolones; for example, the AAC(6')-Ib-cr enzyme increased MIC levels against *E. coli* 4-fold (19). However, PMQR systems are not mutually exclusive and together can confer lower levels of susceptibility to quinolones (1). Transferring *crpP* to *E. coli* J53-3 decreased the susceptibility of this strain to CIP, but not to NOR, MXF, LVX, NAL, or the aminoglycosides KAN, STR, AMK, and GEN. These results indicate that *crpP* encodes a protein that selectively confers resistance to CIP on *E. coli*. The capacity to confer selective resistance to an antibiotic is not uncommon; it is well known that APH enzymes from the same family display variable aminoglycoside resistance profiles (18). APH(3') enzymes phosphorylate KAN and neomycin, but they tend to differ in their specificities for other aminoglycosides (18). Furthermore, AAC(6') enzymes differ in their activities against AMK and GEN C1 (17).

The *in vitro* activity of the purified CrpP enzyme on CIP was determined in order to understand the mechanistic involvement of this enzyme in CIP resistance. CrpP showed enzymatic activity toward CIP and NOR when they were used as substrates. No activity toward LVX, MXF, NAL, or the aminoglycosides KAN and STR was observed, suggesting that CrpP is specific for CIP and NOR. Determination of kinetic parameters indicated that the K_m values for CIP and NOR were similar, although the V_{max} for CIP was >7 times higher than that for NOR. The V_{max}/K_m ratio was approximately 5-fold higher for CIP than for NOR, which could explain why *E. coli* transformed with the *P. aeruginosa* *crpP* gene did not show decreased NOR susceptibility. The APH family includes several members that are differentiated based on their substrate specificities or resistance phenotypes. Thus, members of this family confer resistance to specific antibiotics (20). Similar results were obtained by using the AAC(6')-Ib-cr enzyme, which was more efficient at modifying KAN than CIP, suggesting its different specificities for different antibiotics (8). Moreover, CrpP used ATP as a variable cosubstrate, indicating that the activity of CrpP was ATP dependent. This further suggested that the mechanism underlying CrpP function involves phosphorylation, since APHs catalyze the transfer of a phosphate group to the aminoglycoside molecule, and several of these enzymes use ATP as the donor of the phosphate group (17).

In addition, *in vitro* CIP metabolite analysis using LC-MS-MS suggested that a modification of the CIP molecule occurred, in agreement with the infrared spectroscopy

results. Our analysis revealed the presence of a molecular ion corresponding to CIP-ATP (m/z 820) and several intermediaries of the fragmented CIP-ATP molecule. These analyses enable us to propose that CIP is first activated by binding to the ATP molecule, followed by phosphorylation and degradation. The AAC(6')-Ib-cr protein from *E. coli* is the only CIP-modifying enzyme described to date. This enzyme is a variant of the aminoglycoside acetyltransferase enzyme AAC(6')-Ib, an acetyltransferase that confers resistance to aminoglycosides such as KAN, AMK, and tobramycin (8). The AAC(6')-Ib-cr protein confers reduced susceptibility to CIP by decreasing the activity of this antibiotic via N-acetylation at the amino nitrogen on its piperazinyl substituent (8). Therefore, CrpP may represent a novel protein with specific enzymatic activity against CIP.

In summary, our results show that *crpP* encodes a novel protein capable of specifically conferring resistance to CIP in *E. coli* through an ATP-dependent mechanism that involves phosphorylation of the antibiotic.

MATERIALS AND METHODS

Bacterial strains and culture media. *P. aeruginosa* strain PAO1 (21) and *E. coli* strains JM101 (22) and J53-3 (F⁻ Met⁻ Pro⁻ Rif^r) (23) were used as recipient strains for recombinant plasmids or susceptibility tests. The *P. aeruginosa*(pUM505) strain, expressing the pUM505 plasmid (Hg^r Cr^r) (24), was used to evaluate quinolone resistance, as well as representing a DNA donor of pUM505. The *E. coli* BL21-CodonPlus(DE3)-RP strain (Stratagene, San Diego, CA, USA) was used for protein overexpression. The following culture media were used: nutrient broth (NB), Luria-Bertani (LB) broth (for solid media, 1.5% agar was added) (25), and M9 minimal salts medium (Sigma, St. Louis, MO, USA) supplemented with 20 mM glucose, 0.1 mM CaCl₂, 2.0 mM MgSO₄, and 0.1% casein peptone. When necessary, ampicillin (AMP) (100 µg/ml) or carbenicillin (CAR) (400 µg/ml) was added to agar plates.

Bacterial growth and susceptibility tests. Bacterial cultures were obtained by diluting overnight cultures 1:50 in tubes containing 4 ml of fresh medium. The MICs for CIP, LVX, NOR, MXF, and aminoglycosides were obtained by broth microdilution in Mueller-Hinton medium according to the CLSI recommendations (26). The *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 strains were used as reference strains for antibiotic susceptibility testing.

Cloning of *crpP*. The *crpP* gene was PCR amplified from the pUM505 plasmid using the following oligonucleotides: forward, 5'-CAACATGATGAATTCACCGAAAC-3'; reverse, 5'-GAGAAATGAAGCTTGCGTTGTT-3' (designed with EcoRI and HindIII endonuclease sites [underlined], respectively). The PCR fragment was purified, cloned into the pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA), and transferred to *E. coli* strain JM101 by electroporation; the transformants were selected on LB agar plates containing AMP. The DNA fragment containing *crpP* was obtained by digestion with EcoRI and HindIII and was subcloned into the corresponding sites of the pUCP20 shuttle vector (27). The resulting recombinant pUC-*crpP* plasmid was transferred to the *P. aeruginosa* PAO1 and *E. coli* J53-3 strains by electroporation, and transformants were selected on LB agar plates using CAR or AMP, respectively.

RT-qPCR assays. Total RNA from bacterial cells grown in LB medium was isolated by utilizing the Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). After treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA), RNA was quantified by spectrophotometric analysis at 260 nm. Oligonucleotide primers and hydrolysis probes for RT-qPCR of the *crpP* gene and the 16S rRNA reference genes (listed in Table S1 in the supplemental material) were designed employing Biosearch Technologies software and were purchased from Biosearch Technologies (Novato, CA, USA). The *crpP* and 16S rRNA genes were amplified using the 5' exonuclease probe RT-qPCR method. RT-qPCR was performed with total-RNA samples (500 ng) and the SuperScript III Platinum One-Step RT-qPCR reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) on the LightCycler 480 II system (Roche Molecular Diagnostics, Pleasanton, CA, USA). Amplification signal curves were analyzed at absorption wavelengths of 530 nm. Appropriate positive and nontemplate controls were included in each test run. The relative expression of the *crpP* gene was normalized to expression values obtained from 16S rRNA genes from *P. aeruginosa* (GenBank accession number [AE004091](#); region, nucleotides 722096 to 723631) or *E. coli* (GenBank accession number [AJ605115](#)). Relative gene expression was estimated via the classical calibration dilution curve and slope calculation. A 5-fold dilution series (500 to 0.05 ng total RNA) was prepared and was utilized as samples in the RT-qPCR. Efficiency (*E*) was obtained from standard curves utilizing the formula $E = (1 - 1/\text{slope} - 1) \times 100$. Relative expression levels were determined with the efficiency correction method, which takes into account amplification efficiencies between target and reference genes (28).

Genetic techniques and sequence analysis. General molecular genetics techniques were used according to standard protocols (25). The cloning process was verified by DNA sequencing and was conducted at Elim Biopharmaceuticals, Inc. (Hayward, CA, USA). Protein sequences were aligned using the ClustalW2 package (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Sequence similarities in protein and DNA databases were searched using the blastp and blastx programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Potential promoter sequences were searched using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html).

Overexpression and purification of CrpP. The *crpP* coding region was PCR amplified using pUM505 plasmid DNA as a template and the 5'-ACGAGGGATCCTGTGTCAAAG-3' forward and 5'-AGCGGGATCAAGCTTAAATCGA-3' reverse oligonucleotides, designed with BamHI and HindIII endonuclease sites (underlined), respectively. The amplified fragments were cloned into the pJET1.2/blunt vector, and the

recombinant plasmids were transferred by electroporation into *E. coli* strain JM101; transformants were selected on LB agar plates using AMP. The *crpP* coding region was obtained by digestion with the BamHI and HindIII enzymes, purified, and subcloned into the corresponding restriction sites of the pTrcHisC vector (Thermo Fisher Scientific). The recombinant pTrcHis-*crpP* plasmid encodes the His-CrpP recombinant protein of ≈ 11 kDa, possessing a 6 $\times$ His tag at the N terminus. The clones were validated by sequencing with the pTrcHis forward primer (Thermo Fisher Scientific). The recombinant plasmids were transferred by electroporation into the *E. coli* BL21-CodonPlus(DE3)-RP strain, and transformants were selected on LB agar plates using ampicillin. Overnight cultures of *E. coli* BL21(pTrcHis-*crpP*) were diluted at a 1:50 ratio into 250 ml of fresh NB medium and were cultured at 37°C with shaking until the optical density at 590 nm (OD_{590}) reached 0.5. Overexpression of the His-CrpP protein was induced with 0.5 mM IPTG, and the culture was incubated for 6 additional hours. Cells were harvested by centrifugation, and the pellets were suspended in a loading buffer composed of 25 mM Tris (hydroxymethyl) amino methane-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 0.35 mM β -mercaptoethanol, 12.5% glycerol, and 0.005% bromophenol blue, after which they were heated for 5 min at 95°C. His-CrpP overexpression was verified on a 17% SDS-PAGE gel using a Tris-glycine buffer.

His-CrpP protein was purified as described by Watanabe and Takada (29). Cells were disrupted by sonication until the solution was clarified, and the debris was removed by centrifugation. The supernatant was loaded onto a nickel-nitrilotriacetic acid (NTA) resin (Qiagen, Venlo, The Netherlands) and was packed into a column. His-CrpP was recovered by elution with 200 mM imidazole, as described by Aranda et al. (30). The purification was monitored by 17% SDS-PAGE.

Phosphorylation assays. Phosphorylation of quinolones (CIP, NOR, LVX, MXF, and NAL) or aminoglycosides (KAN and STR) by CrpP was monitored with a coupled assay using pyruvate kinase-lactate dehydrogenase, as described by Kramer and Matsumura (16), with the following modifications. We incubated 0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 mM quinolone with 5.0 μ g/ml of His-CrpP protein in a total volume of 1 ml of assay buffer (50 mM Tris [pH 7.6], 40 mM KCl, 10 mM $MgCl_2$, 0.25 mg/ml NADH, 2.5 mM phosphoenolpyruvate, and 2.0 mM ATP) and 5.0 μ l of pyruvate kinase-lactate dehydrogenase (600 to 1,000 μ g/ml pyruvate kinase or 900 to 1,400 μ g/ml lactate dehydrogenase [Sigma]) in a quartz cuvette (catalog no. 14-385-914A; Thermo Fisher). The mixtures were incubated at 37°C for 15 min. The oxidation of NADH was determined by monitoring the absorbance at 340 nm with an Amersham Biosciences Ultrospec 4300 pro UV/visible spectrophotometer (Amersham, UK). In this coupled assay system, the amount of ADP released is monitored at 340 nm through coupling with NADH oxidation. The data were plotted using nonlinear regression, the Michaelis-Menten model, and the least-squares method. GraphPad Prism software, version 5.01 for Windows, was used. Additionally, the activity of the His-CrpP protein on CIP was analyzed after its denaturation by heating at 95°C for 15 min, and the oxidation of NADH was determined as described above. To determine the optimal pH for CrpP activity, the pH of the reaction mixture was adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0. Moreover, to analyze the effect of the Mg^{2+} concentration on CrpP activity, the $MgCl_2$ concentration in the reaction mixture was modified to 5.0, 10.0, or 15.0 mM. Next, 0.5 mM fluoroquinolone was incubated with 5.0 μ g/ml of His-CrpP protein in a total volume of 1 ml for each reaction mixture, and the oxidation of NADH was determined. Six independent assays with two replicates per experiment were conducted.

Analytical methods. To determine whether CIP is modified by CrpP, LC-MS-MS analysis was conducted. Thus, 5.0 mM CIP was incubated with 5.0 μ g/ml of His-CrpP protein for 1 h at 37°C, in the presence or absence of 2.0 mM Mg-ATP (Sigma), in buffer R (50 mM HEPES [pH 7.0], 40 mM KCl, 10 mM $MgCl_2$) in a total volume of 1 ml. Next, the samples were centrifuged at 13,700 $\times g$ and were filtered using a 0.2- μ m Millipore (Billerica, MA, USA) membrane. The LC-MS-MS system consisted of an Acquity ultraperformance liquid chromatography (UPLC) column and a Xevo TQ-S instrument (Waters, Milford, MA, USA).

LC-MS-MS measurement was carried out as follows. One microliter of the standard solution or previously filtered samples was injected into a reversed-phase LC column (Acquity UPLC BEH C_{18} column; particle size, 1.7 μ m; inside diameter, 2.1 mm; length, 50 mm). CIP was eluted from the column using the following conditions: water containing 0.01% formic acid (A) and 100% acetonitrile (B). Elution was begun with 100% A–0% B followed by a linear gradient to 90% A–10% B (until 0.25 min), followed by 40% A–60% B (until 3.50 min), and finally by 100% A–0% B (until 4.50 min) plus 1 min for equilibration. A flow rate of 0.4 ml/min was used. A column temperature of 40°C was maintained, while the temperature of the samples in the autosampler was 10°C. For detection, a Xevo TQ-S instrument was used in the scan and multiple-reaction monitoring (MRM) modes. Scanning was carried out from 30 to 1,000 m/z with a 0.2-s scan time, a 40-V cone voltage, in the positive-ionization mode. For the MRM mode, the specific parameters for the MS-MS transition were as follows. For CIP transition, there was a precursor ion of m/z 332.2 and product ions of m/z 245.2, 288.3, and 314.2 with collision energies of 25, 15, and 20 V, respectively. The cone voltage (V) was 5 V, and the dwell time was 0.020 s. All three transitions were measured using a capillary voltage of 2.20 kV, a desolvation temperature of 500°C, a desolvation gas flow (N_2) of 800 liters/h, a cone gas flow of 50 liters/h, and a nebulizer gas flow of 7.0×10^5 Pa. The calculations were performed using the ratios of the peak areas of the quantified transition components of CIP.

Additionally, samples obtained under the conditions described above were analyzed by infrared spectroscopy, and the spectra were recorded using a Nicolet iS10 spectrophotometer (Thermo Scientific) and the attenuated total reflection (ATR) technique (31).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02629-17>.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.**ACKNOWLEDGMENTS**

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Original Research Article

A plasmid-encoded mobile genetic element from *Pseudomonas aeruginosa* that confers heavy metal resistance and virulence

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ABSTRACT

Mobile plasmid-encoded elements are DNA segments that are transferred for horizontal gene transfer and that confer adaptive properties, as well as virulence and antibiotic and heavy metal resistance to bacteria. The conjugative plasmid pUM505, isolated from a clinical strain of *Pseudomonas aeruginosa*, possesses a putative 31.292 kb mobile element (denominated Mpe: Mobile plasmid- encoded element) that, in addition to possessing *chr* genes that confer chromate resistance to *Pseudomonas*, contains two putative *mer* operons that could confer mercury resistance. Moreover, the Mpe contains genes related previously with the virulence of both *P. aeruginosa* and *Escherichia coli* strains. In this work, we determined that Mpe from pUM505 was able to independently move to another DNA molecule, conferring chromate and mercury resistance to *P. aeruginosa* PAO1 and mercury resistance to *E. coli* JM101, suggesting that its transference might be beneficial to bacteria under certain environmental conditions. Additionally, the transference of Mpe increased the virulence of *P. aeruginosa* PAO1 against the nematode *Caenorhabditis elegans*, suggesting its contribution to the pathogenicity of *P. aeruginosa*. In this work, we describe a new mobile plasmid-encoded element that possesses the potential to be transferred by horizontal gene transference, which could provide bacteria with a wide variety of adaptive traits such as heavy metal resistance and virulence, 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

Bacterial species can evolve through different genomic modifications that include mutations, rearrangements, or horizontal gene transfer (HGT) (Juhas et al., 2009). HGT is a phenomenon directed by mobile genetic elements (MGE) such as plasmids, insertion sequences, integrons, transposons, phages, and DNA segments recognized as genomic islands (GI) (Stokes and Gillings, 2011). MGE allow variation between closely related bacterial strains and confer adaptive properties such as virulence, antibiotic, and heavy-metal resistance, xenobiotic-compound degradation, and the formation of new metabolic pathways (Juhas et al., 2009; Haritha et al., 2009). Many bacterial genes that encode virulence factors such as adhesins, secretion systems, invasins, toxins, and iron uptake systems are located within GI, which are denominated pathogenicity islands (PAI) (Ho Sui et al., 2009; Che et al., 2014). Plasmids, which additionally encode a variety of traits including

drug resistance and the metabolism of rare substances, are able to promote the dissemination of virulence (Funnell and Phillips, 2004; Rodríguez-Andrade et al., 2016). In *Cupriavidus metallidurans* and *Pseudomonas putida* KT2440, putative GI containing metal resistance genes were found to be related with the evolution of metal homeostasis, providing support for the prominent role of HGT in this process in proteobacteria (Haritha et al., 2009). In nosocomial bacteria selected by the use of antibiotics during therapeutic procedures, it has been reported that, in addition to possessing genes that confer antibiotic resistance, genes for resistance to heavy metals may also be present as GI or may form part of plasmids (Caballero-Flores et al., 2012). Mercury-resistance operons are the most frequent heavy metal resistance genes reported to be associated with GI and plasmids (Reva and Bezuidt, 2012). Bacterial resistance to hexavalent chromium (chromate; CrO₄²⁻ or Cr) has been reported mainly in environmental bacteria, including Gram-positive and Gram-negative strains (reviewed in Ramírez-Díaz

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et al., 2008). In conjugative plasmids with the co-occurrence of heavy metal and antibiotic resistance genes, metal exposure can also promote the HGT of antibiotic resistance, which has become a major health challenge in clinical settings (Pal et al., 2015). Moreover, it has been reported that plasmids with antibiotic and heavy-metal resistance genes that also possess a Toxin-Antitoxin (TA) system are likely to be more persistent in the absence of selection pressure, compared with resistance plasmids that lack a TA system, since TA systems stabilize plasmids in their hosts by killing daughter cells that do not inherit the plasmids with the TA system (Pal et al., 2015).

pUM505 from *Pseudomonas aeruginosa* is a conjugative plasmid of 123 kilobases (kb) that possesses genetic determinants involved in resistance to the oxyanion Cr, and two putative *mer* operons that could confer resistance on the heavy metal, mercury (Hg), grouped on a 31.292 kb gene cluster (RefSeq accession number NC_016138; region, nucleotides 47,807–78,956) (Ramírez-Díaz et al., 2011). This region also contained several features frequently found associated with GIs and MGEs plasmid-encoded, including the presence of flanking repeats, mobility genes (e.g., genes encoding integrases, resolvases, and transposases), and insertion sequence (IS) elements (Ramírez-Díaz et al., 2011). By in silico analysis, we determined that the Hg and Cr resistance genes of the 31.292 kb element encoded by pUM505 are distributed over three putative transposable elements: the first of these contains an incomplete *mer* operon, constituted of *merRTP* genes flanked by a *tnpA* gene and by a gene that encodes a putative recombinase; the second is constituted of *merRTPFADE* genes that are flanked by *bin* and *tnpA* genes, which encode resolvase and a putative type TnpA transposase, respectively, while the third transposon contains *chrABC* genes flanked by genes encoding transposase/integrase TnpA and resolvase/integrase TnpR (Ramírez-Díaz et al., 2011). In addition to the Cr and Hg resistance genes, it has been found that the 31.292 kb element from pUM505 possesses genes that encode proteins that increased the virulence of *P. aeruginosa* PAO1 in mice and decreased the viability of HeLa cells, suggesting that these genes encode virulence factors (Rodríguez-Andrade et al., 2016). It was recently reported that pUM505 possesses the *crpP* gene that encodes a new mechanism of resistance to Ciprofloxacin in *P. aeruginosa*, which involves phosphorylation of the antibiotic (Chávez-Jacobo et al., 2018), suggesting the importance of antibiotic resistance genes in the prevalence of the plasmid. Furthermore, pUM505 possess a functional TA system that confers plasmid stability, and this toxin possesses the potential to increase *P. aeruginosa* virulence (Hernández-Ramírez et al., 2017), suggesting that this TA system could increase the persistence of pUM505 in *Pseudomonas* in the absence of selection pressure.

In the present study, we determined the self-mobility of the 31.292 kb element encoded by pUM505 that carried heavy metal resistance and virulence genes, suggesting that this mobile element could have been acquired by HGT and might be a factor for the prevalence and distribution of this *P. aeruginosa* plasmid.

2. Materials and methods

2.1. Bacterial strains, culture media, and vectors

P. aeruginosa wild type strain PAO1, obtained from Dr. B. Iglewski's collection (Li et al., 2007) and *Escherichia coli* strain JM101 (Yanisch-Perron et al., 1985) were used as recipient strains for plasmids and susceptibility tests. *P. aeruginosa* PAO1 (pUM505), Cr and Hg resistant (Cr^R and Hg^R, respectively) (Cervantes et al., 1990), contains the pUM505 plasmid and donated the 31.292 kb pUM505 encoded-element (called Mpe from mobile plasmid-encode element). *E. coli*/*Pseudomonas* shuttle vector pUCP20, which contains a Carbenicillin resistance gene (West et al., 1994) was used as a recipient of Mpe.

The culture media consisted of the following: Nutrient Broth (NB) (Bioxon, Franklin Lakes, NJ, USA); Luria-Bertani broth (LB), and M9 salts minimal medium (Sigma, St. Louis, MO, USA) supplemented with

20 mM glucose and 0.1 mM CaCl₂. A total of 1.5% agar was added to obtain solid medium. Where necessary, Carbenicillin (Cb) (400 µg ml⁻¹) was added.

2.2. Bacterial growth and susceptibility tests

For determination of Minimal Inhibitory Concentrations (MIC), bacterial cultures were grown overnight in LB broth and diluted 1:100 in fresh medium. A total of 25 µl drops were inoculated on LB agar plates with or without the addition of increasing concentrations of K₂CrO₄ (25–200 µM) or HgCl₂ (0.005–50 µM) (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 24 h at 37 °C. The MIC was defined as the lowest concentration of the compound that completely inhibited bacterial growth.

Cr and Hg susceptibility tests in liquid cultures were performed as follows: bacteria were grown by diluting 1:100 overnight cultures in tubes containing 4 ml of NB with increasing amounts of K₂CrO₄ or HgCl₂. Cultures were incubated for 18 h at 37 °C with shaking, and growth was measured as the Optical Density (OD) at 590 nm (OD₅₉₀) with a spectrophotometer.

2.3. Transfer of Mpe and screening

In order to test the ability of the Mpe of pUM505 to be transferred to other DNA molecules, the following strategy was employed: First, the pUCP20 vector was transferred by electroporation into *P. aeruginosa* PAO1 (pUM505) and transformants were selected on LB agar plates with Cb. Thereafter, to confirm the presence of the pUM505 and pUCP20 plasmids in the transformants, the DNA of both plasmids were isolated by the Casse method (Casse et al., 1979) from NB-grown overnight cultures from *P. aeruginosa* (pUM505) (pUCP20) (Cr^R, Hg^R, Cb^R) colonies and were analyzed by electrophoresis. Cultures of 4 ml of *P. aeruginosa* (pUM505) (pUCP20) were grown in LB at 37 °C for 18 h without Cb. Then, 100 µl of bacterial dilutions (1 × 10⁻⁷ to 1 × 10⁻⁹) in fresh medium were inoculated on Nutrient Agar (NA) plates to obtain isolated colonies. Approximately 10,000 colonies were inoculated simultaneously on NA plates and NA plates supplemented with K₂CrO₄ (150 µM), HgCl₂ (0.02 µM), or Cb, respectively. Mutant colonies of *P. aeruginosa* (pUM505) (pUCP20) with a Cr^R, Hg^R, and Cb sensitive (Cb^S) phenotype, generated from the insertion of Mpe into the gene for Cb resistance in the pUCP20 vector, were selected. The recombinant plasmid was designated as pUCP20-Mpe.

2.4. Molecular characterization of the recombinant pUCP20-Mpe plasmid

The pUCP20-Mpe plasmid was isolated from *P. aeruginosa* (pUM505) (pUCP20) Cr^R, Hg^R, and Cb^S mutant colonies using the alkaline lysis protocol (Birnboim and Doly, 1979) with some modifications; this protocol was used in that it does not allow the isolation of the pUM505 plasmid. The pUCP20-Mpe plasmid was then transferred by electroporation to *P. aeruginosa* strain PAO1 and the transformants were selected by culturing on LB agar with K₂CrO₄ 150 µM. Next, the pUCP20-Mpe plasmid was isolated from cultures of *P. aeruginosa* (pUCP20-Mpe) (Cr^R) transformants and analyzed by gel electrophoresis employing the 1 kb-plus DNA ladder (Life Technologies, Rockville, MD, USA) as a marker. The presence of Mpe in the pUCP20-Mpe plasmid was analyzed by the amplification of *chrA* and *merA* from pUM505 by PCR. The *chrA* gene, including its 5' putative regulatory region, was amplified using the following primers: ChrA_D 5'-GTTACGAGGATCCA ACAACGTGAACGCCCG-3' (forward) and ChrA_R 5'-TGGCTTCTGCA GCAAGGTGGATCGGAAACG-3' (reverse) (predicted fragment size, 1.5 kb), and an internal region from the *merA* gene was amplified using the primers MerA_D 5'-CATATCGCCATCATTTGGCAGC-3' (forward) and Mer_R 5'-CCTCGATGACCACTTGATGAAG-3' (reverse) (predicted fragment size, 1.2 kb).

Additionally, susceptibility tests of the *P. aeruginosa* PAO1 (pUCP20-

Mpe) transformants into K_2CrO_4 and $HgCl_2$ were conducted. Determination of the site of insertion of the Mpe into the pUCP20 vector was determined by DNA sequencing of pUCP20-Mpe conducted by Elim Biopharmaceuticals, Inc. (Hayward, CA, USA).

2.5. Virulence assays

Caenorhabditis elegans Bristol N2 (Brenner, 1974) worms were synchronized by hypochlorite isolation of eggs from gravid adults, followed by the hatching of eggs in S-basal medium. L1 larvae were transferred onto NGM (Nematode Growth Medium) (Stiernagle, 2006) on plates seeded with the *E. coli* OP50 strain previously grown on the plates as a food source and incubated at 20 °C for 4–5 days until they reached the young adult phase. Worms were rinsed off the plates and washed in S-basal medium. Cultures of *P. aeruginosa* were grown overnight in M9 broth at 37 °C, centrifuged, and the pellets were suspended in fresh M9 medium and grown to an $OD_{590} = 0.4$. The worms were incubated with $1 \times 10^5 (\pm 1 \times 10^4)$ Colony-Forming Units (CFU) of each bacterial strain as described by Hernández-Ramírez et al., 2017. *P. aeruginosa* PAO1 (pUCP20) was employed to determine the level of virulence of the PAO1 strain; whereas *P. aeruginosa* PAO1 (pUM505) was utilized as a positive control for increasing virulence. M9 medium was used as negative control. A worm was considered dead when it no longer responded (moved) to a touch stimulus. Worms that died as a result of adherence to the wall of the plate were excluded from the analysis. For all assays, bacterial concentrations were confirmed by dilution, plating, and counting. Two independent assays with two replicates were performed for each worm group.

2.6. Transference of the pUCP20-Mpe plasmid to *E. coli* JM101

The pUCP20-Mpe recombinant plasmid was transferred to *E. coli* JM101 competent cells by electroporation, and transformants were selected by culturing on LB agar with $HgCl_2$ 20 μ M or K_2CrO_4 50 μ M. The pUCP20-Mpe was isolated and *chrA* and *merA* were amplified by PCR using the same primers and conditions employed for the amplification of DNA from *P. aeruginosa* (pUCP20-Mpe) (Cr^R) transformants. Additionally, susceptibility tests of the *E. coli* (pUCP20-Mpe) transformants to K_2CrO_4 and $HgCl_2$ were conducted.

2.7. Genetic techniques and sequence analysis

General molecular genetic techniques were used according to standard protocols (Green and Sambrook, 2012). Protein sequences were aligned using the CLUSTAL W2 software package (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Sequence similarities in protein and DNA databases were analyzed using the blastp and blastx programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Potential promoter sequences were obtained using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html).

2.8. Statistical analysis

Data analysis was performed using GraphPad Prism ver. 5 software. Analysis of variance (ANOVA) employing the Tukey test was performed for all assays. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Transference of Mpe from pUM505

Using in silico analysis, we determined that the conjugative plasmid pUM505 possesses a putative 31,292 kb mobile genetic element that is integrated by the Open Reading Frames (ORF) 59–94 (Ramírez-Díaz et al., 2011), which was called Mpe for mobile plasmid-encoded element (as described in Materials and Methods) (Fig. 1). We investigated

whether the Mpe from pUM505 is self-mobile (able to move to other DNA molecules). To determine this, first, the Minimal Inhibitory Concentration (MIC) for Cr and Hg were performed to obtain the ion concentration able to select for Mpe-positive colonies. The MIC results showed, as expected, that *P. aeruginosa* PAO1 (pUM505) exhibited resistance to Cr and Hg with respect to *P. aeruginosa* PAO1 and *P. aeruginosa* (pUCP20), which were inhibited at 100 μ M of K_2CrO_4 and at 0.01 μ M of $HgCl_2$ (Table S1). Then, pUCP20 was transferred to *P. aeruginosa* PAO1 (pUM505), and one *P. aeruginosa* (pUM505) (pUCP20) transformant with the Cr^R , Hg^R , and Cb^R phenotype was selected. The plasmid DNA was isolated and analyzed by electrophoresis, showing the presence of both plasmids (pUM505 and pUCP20) in the transformant (Fig. S1). From ~10,000 colonies isolated from *P. aeruginosa* (pUM505) (pUCP20) transformant grown in medium without the addition of Cb, we obtained two mutants with a Cr^R , Hg^R , and Cb^S phenotype. To determine whether the Cb^S phenotype of the mutants isolated was conferred by the interruption of the Cb gene of pUCP20 by the transfer of Mpe and to discard that the phenotype was conferred by the loss of the pUCP20 vector, the plasmid DNA from *P. aeruginosa* (pUM505) (pUCP20) Cr^R , Hg^R , and Cb^S mutants was isolated and transferred by electroporation into *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 Cr^R , Hg^R , and Cb^S transformants were obtained and their DNA plasmids were analyzed by electrophoresis. The plasmids isolated from these colonies are smaller than the pUM505 plasmid and larger than the pUCP20 backbone vector of ~3.8 kb (Fig. S2). The isolation of a larger plasmid (respect to pUCP20 vector) suggested that this size increase was due to Mpe mobility into the Cb gene, and for this reason, this plasmid was denominated as pUCP20-Mpe. The presence of the Mpe in the pUCP20 vector was analyzed by PCR amplification of the *chrA* and *merA* genes using plasmid DNA from *P. aeruginosa* PAO1 (pUCP20-Mpe). The results revealed that both of the amplified genes obtained bands of the expected size (Fig. 2a), discarding the possibility that the phenotype of sensitivity to Cb is due to the loss of pUCP20 and that, in turn, resistance to Cr and Hg was conferred by the plasmid pUM505. These results suggested the insertion of Mpe from pUM505 into the pUCP20 vector.

3.2. Transfer of the functional Cr and Hg mechanisms of resistance contained in Mpe

To determine whether the mobility of Mpe confers a functional heavy metal resistance phenotype, the susceptibility of *P. aeruginosa* PAO1 (pUCP20-Mpe) transformant to Cr and Hg was determined. *P. aeruginosa* PAO1 (pUCP20-Mpe) transformant showed increased resistance to both ions compared with the control strain (Fig. 2b). This result indicates that some of the genes contained in Mpe from pUM505 are able to confer heavy metal resistance to *P. aeruginosa* PAO1.

To determine whether the genes encoded in the Mpe of pUM505 are able to be functional in the heterologous bacterial host, the pUCP20-Mpe plasmid was transferred to *E. coli* JM101. The results showed that *E. coli* JM101 (pUCP20-Mpe) transformants only showed resistance to Hg, but not to Cr (Fig. 3a). The presence of *chr* resistance genes in the pUCP20-Mpe plasmid isolated from *E. coli* JM101 (pUCP20-Mpe) transformants was analyzed by PCR amplification of the *chrA* gene. The results showed a band of the size expected for *chrA* when the plasmid DNA from *E. coli* JM101 (pUCP20-Mpe) transformants was used as a template (Fig. 3b), indicating that the *chr* resistance operon was present and additionally suggesting that the system of resistance to Cr is not functional in *E. coli*. However, the resistance to Hg of *E. coli* JM101 (pUCP20-Mpe) transformants indicating that Mpe has the potential to provide heavy metal resistance mechanisms to bacteria other than *Pseudomonas*.

3.3. Mpe increases the virulence of *P. aeruginosa* PAO1

Because the Mpe contains genes (*orf*s 92, 93, and 94) previously

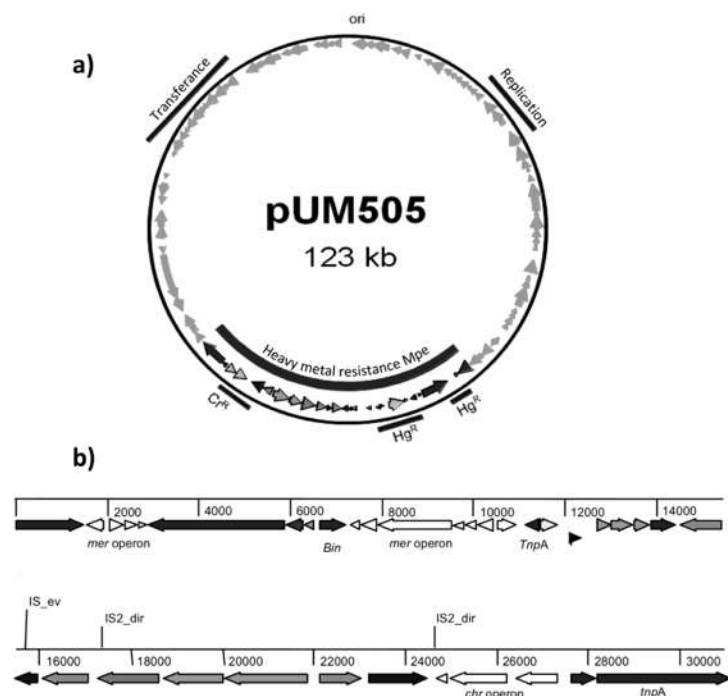


Fig. 1. Schematic representation of the heavy-metal resistance and virulence mobile plasmid-encoded element of the pUM505 (Mpe). Deduced coding regions are shown by arrows or arrowheads indicating the direction of transcription. a) Localization of the heavy metal resistant Mpe in the pUM505 plasmid is indicated in light gray. Putative operons containing coding regions predicted to be involved in conjugative transfer (transference), replication (replication), mercury resistance (Hg^R), and chromate resistance (Cr^R) are indicated by dark gray bars. b) Genetic map of Mpe. The image shows the genetic organization of the genes contained in Mpe. Mercury (*mer*) and chromate (*chr*) resistance determinants are shown with white arrows. Putative mobile elements present in transposons are shown in black and the putative insertion sequence elements characteristic of the Tn3-like transposons family are shown by black vertical lines (IS).

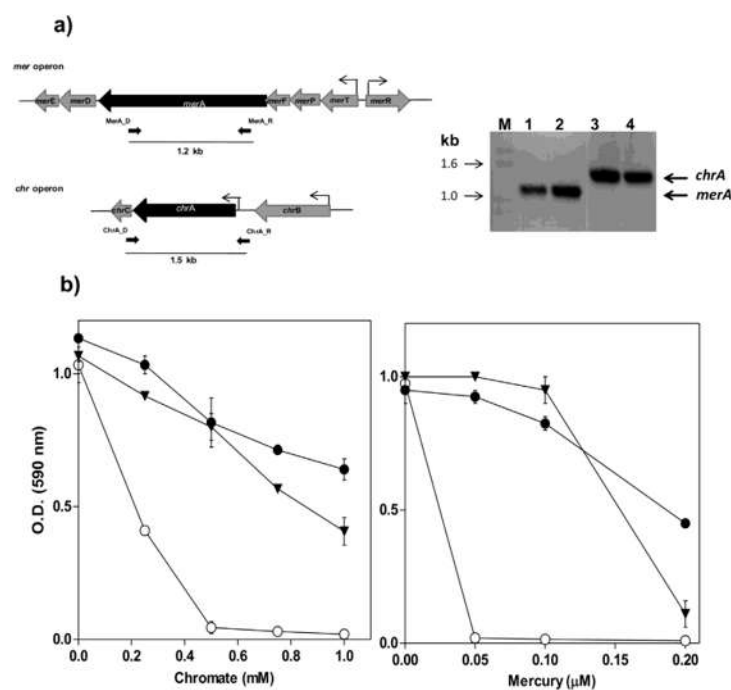


Fig. 2. Heavy metal resistance conferred by Mpe to the *P. aeruginosa* PAO1 strain. a) Molecular confirmation through PCR show the *mer* and *chr* resistance genes in *P. aeruginosa* PAO1 (pUCP-Mpe). On the left, the diagram shows the localization of the primers used for the PCR amplification of *mer* and *chr* genes. On the right, the PCR amplicons of 1.2 and 1.5 kb, respectively, of *merA* (lanes 1 and 2) and *chrA* (lanes 3 and 4) genes from *P. aeruginosa* PAO1 (pUM505) (lanes 1 and 3) or PAO1 (pUCP-Mpe) (lanes 2 and 4) strains are shown. M, Molecular size markers (in kilobases). c) Susceptibility to chromate and mercury of the *P. aeruginosa* PAO1 (pUCP-Mpe) transformant. Strains were grown in NB for 18 h at 37 °C with shaking and with the indicated concentrations of potassium chromate and mercury chloride. Growth was measured as OD at 590 nm. *P. aeruginosa* PAO1 (pUCP20) (○), PAO1 (pUM505) (●), PAO1 (pUCP-Mpe) (▼). Data shown are the means of three independent assays, in duplicate with Standard Deviation (SD) bars shown.

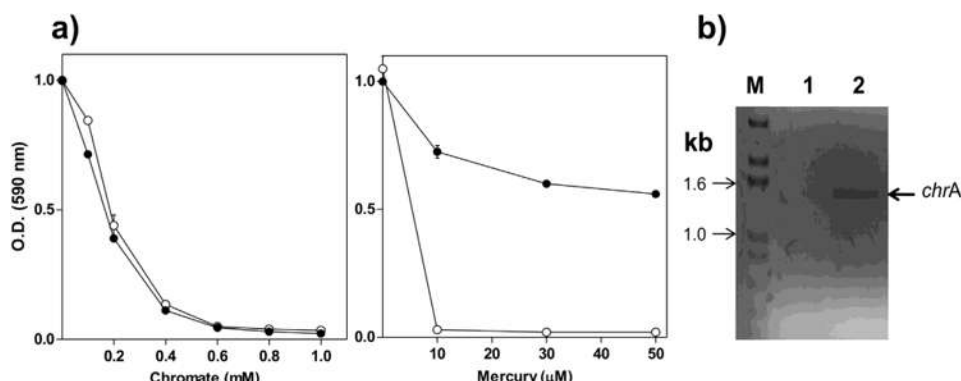


Fig. 3. Heavy metal resistance conferred by Mpe to the *E. coli* JM101 strain. a) Susceptibility to chromate and mercury of *E. coli* JM101 (pUCP20-Mpe) transformant. Strains were grown in NB for 18 h at 37 °C with shaking and with the indicated concentrations of chromate or mercury. Growth was measured as OD at 590 nm. *E. coli* JM101 (pUCP20) (○) and *E. coli* JM101 (pUCP-Mpe) (●). Data shown are the means of three independent assays, in duplicate with Standard Deviation (SD) bars shown. b) Molecular characterization to show the *chrA* resistance gene of the pUCP20-Mpe transferred to *E. coli* JM101. Line 1, PCR from the *E. coli* JM101 (pUCP20) strain; lane 2, PCR amplification from *E. coli* JM101 (pUCP20-Mpe) transformant. M, Molecular size markers (in kilobases).

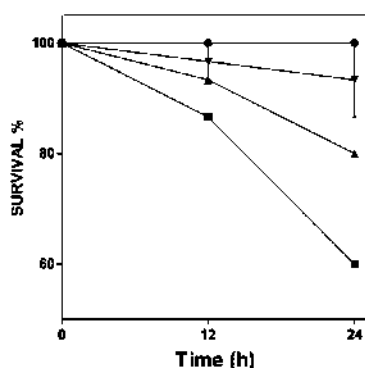


Fig. 4. Influence of Mpe on the virulence rate. Groups of 20 worms were incubated with 1×10^5 ($\pm 1 \times 10^4$) Colony-Forming Units (CFU) of the indicated strains, as described under Materials and Methods. Mixtures were incubated at 20 °C and scored for live worms at the indicated times. Symbols: (●), M9 medium; (▼), *P. aeruginosa* PAO1 (pUM505); (▲), *P. aeruginosa* PAO1 (pUCP20); and (■), *P. aeruginosa* PAO1 (pUCP-Mpe). The data shown are means from the duplicates of two independent assays.

related with *P. aeruginosa* virulence (Rodríguez-Andrade et al., 2016), we investigated whether the mobility of this mobile plasmid-encoded element could increase the virulence of the bacterial host. The results showed that *P. aeruginosa* (pUM505) killed 40% of the nematodes at 24 h post-incubation (Fig. 4), which was expected, since pUM505 contains several virulence genes located in a pathogenic island (Rodríguez-Andrade et al., 2016; Hernández-Ramírez et al., 2017). Interestingly, *P. aeruginosa* (pUCP20-Mpe) killed 20% of the worms at 24 h post-incubation, whereas *P. aeruginosa* (pUCP20) killed only 6% of the worms at the same timepoint (Fig. 4). These data demonstrate that the Mpe of pUM505 possesses functional genes that are important for increasing the virulence of *P. aeruginosa*.

3.4. Identification of the 5' and 3' end regions and sequence analysis of Mpe from pUM505

To determine whether the 5' and 3' end regions of the Mpe from

pUM505 were present in the pUCP20-Mpe plasmid, it was sequenced. Sequence analyses established that Mpe begins with ORF 59 and ends with ORF 94 from pUM505 (NCBI Reference Sequence: NC_016138), as predicted by in silico analysis (Ramírez-Díaz et al., 2011). Also, we determined that Mpe was inserted between nucleotides 2033 and 2900 of the pUCP20 vector, located in the Cb resistance gene, which confirms that the loss of Carbenicillin resistance is due to the insertion of Mpe in the beta-lactamase gene of pUCP20 vector. Additionally, the sequence of the pUCP-Mpe plasmid demonstrated that the *mer* and *chr* genes do not change once that Mpe was transferred, as well as that no additional genes outside of Mpe were transferred.

In addition, a detailed analysis of the Mpe sequence revealed that the region comprising ORF 84 and 89 possesses 99% identity with a genome region (from nucleotides 3,080,172–3,088,798) located in the genomic island GI7 from *P. aeruginosa* DK2 (Fig. 5). *P. aeruginosa* DK2 (access number CP003149) is a clone with a history of infecting Cystic Fibrosis (CF) patients (Yang et al., 2011). ORF 85, 86, and 87 of Mpe encode hypothetical proteins. ORF 84 and 88 of Mpe encode putative AraC transcriptional regulators (Fig. 5), a family of transcriptional regulators that are widespread among bacteria and that are related with stress responses and virulence (Gallegos et al., 1997). ORF 89 of Mpe encodes a putative peptidase of the M16 family (Fig. 5) (Aleshin et al., 2009). This analysis suggests that accessory functions are encoded by pUM505.

4. Discussion

The 123 kb conjugative plasmid pUM505 was isolated from a *P. aeruginosa* clinical strain (Cervantes et al., 1990; Ramírez-Díaz et al., 2011). The Mpe from the pUM505 plasmid contains features frequently found to be associated with MGE, as well as flanking repeats, mobility genes, and IS elements containing genetic determinants involved in Cr and Hg resistance and virulence (Ramírez-Díaz et al., 2011; Rodríguez-Andrade et al., 2016). With the aim of determining whether the Mpe of 31.292 kb from pUM505 is able to self-mobilize to other DNA molecules, we carried out a genetic approach using mutants that had lost the Cb resistance phenotype of pUCP20 vector. The results showed that Mpe self-mobilized to the pUCP20 vector and conferred resistance to Hg in *P. aeruginosa* and *E. coli* strains, suggesting that the mechanism of Hg resistance encoded by the genes of Mpe could participate in the adaptability of the bacterial host in environments contaminated by metals. Also, the self-mobility of Mpe to pUC20 conferred Cr resistance

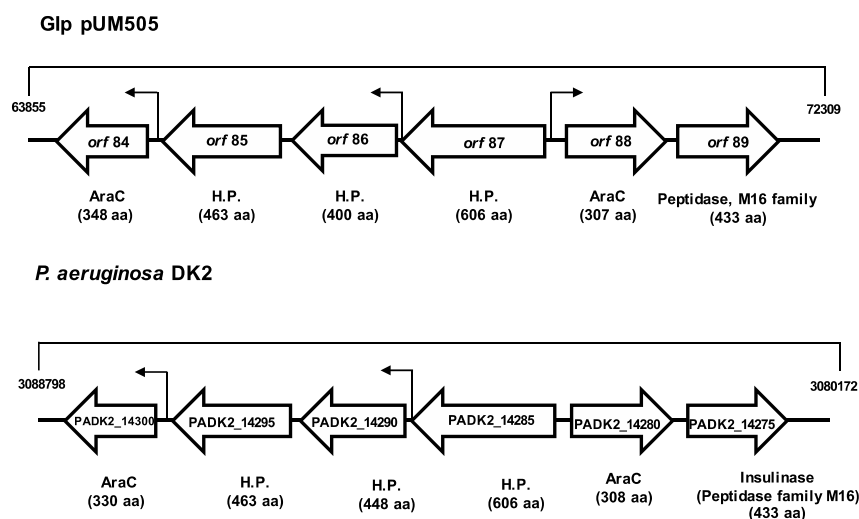


Fig. 5. Genetic organization of the regions conserved between Mpe of pUM505 and GI7 of *P. aeruginosa* DK2. Deduced coding regions are shown by arrows indicating the direction of transcription. The size in amino acids (aa) and the putative function of the encoded proteins are indicated, as well as Hypothetic Protein (HP). Locations of putative promoters are indicated by small arrows (r).

on *P. aeruginosa* PAO1; however, it did not confer Cr resistance on *E. coli*. This result was not surprising, since Cervantes et al. (1990) reported that the protein encoded by the *chrA* gene from pUM505, which is sufficient to confer resistance on *P. aeruginosa*, does not confer Cr resistance on *E. coli*, suggesting a transcriptional barrier or the incapacity of protein ChrA to function in *E. coli*. To determine that *chr* genes were not lost during the self-mobility of Mpe, the *chrA* gene was amplified from *E. coli* JM101 (pUCP-Mpe) transformants by PCR. Amplification of the *chrA* gene indicated that the *chr* genes were not lost in Mpe. Also, the DNA sequence of Mpe show that the *merA* and *chrA* genes dot not change after that this mobile plasmid-encoded element was transferred to another DNA molecule. These data indicate that, although resistance to the chromate conferred by Mpe is not functional in *E. coli*, the *chr* genes transferred may be important for the survival of *Pseudomonas* strains in sites contaminated by this metal.

These results indicate that Mpe has the potential to provide heavy metal resistance mechanisms to bacteria, suggesting that its transference might be beneficial to bacteria under certain environmental conditions.

We aimed to determine whether the Mpe was able to increase the virulence of *P. aeruginosa* against the nematode *Caenorhabditis elegans*, potentially via the function of products encoded by *orfs* 92, 93, and 94, which were previously related with virulence (Rodríguez-Andrade et al., 2016). The results showed that Mpe contributed to the pathogenicity of *P. aeruginosa*, demonstrating an increased death rate of the worms compared with the strain without the Mpe element. Computational approaches of analyzing pathogenic bacterial genomes have revealed that the pathogenic genomic regions, or PAIs, were horizontally transferred from other bacteria (Che et al., 2014), suggesting that these genes are important for conferring traits of virulence on the bacterial host.

Finally, the pUM505 Mpe was shown to contain a region that harbors ORF 84–89, conserved in *P. aeruginosa* DK2, a clone that emerged as a highly successful colonizer of the CF lung (Yang et al., 2011). These data suggest that the putative accessory functions encoded by Mpe from pUM505 could be important for ensuring the proliferation of *Pseudomonas* strains; however, a functional analysis is required.

In summary, in this study, we determined that the pUM505 Mpe

plasmid has the capacity to self-mobilize to other DNA regions, suggesting that it is a functional mobile genetic element that could contribute to the transference of novel genes. These novel genes may confer a variety of adaptive properties to *P. aeruginosa* and possibly to other pathogenic bacteria, including, *E. coli*, such as heavy metal resistance systems and virulence, which may be involved in evolution and pathogenesis.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plasmid.2018.07.003>.

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Prevalence of the *crpP* gene conferring decreased ciprofloxacin susceptibility in enterobacterial clinical isolates from Mexican hospitals

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Objectives: This study investigated the presence of the *crpP* gene, which encodes an enzymatic mechanism of antibiotic phosphorylation that decreases ciprofloxacin susceptibility, in ESBL-producing clinical isolates and its effect in transconjugants.

Methods: A collection of 77 ESBL-producing clinical isolates of Enterobacteriaceae and 68 ESBL-producing transconjugants that had acquired plasmids from clinical isolates from hospitals in Mexico obtained from 1988 to 2012 was employed. The *crpP* homologue genes were identified by dot-blot and PCR assays; five of them were sequenced and an *in silico* analysis was conducted. Expression of CrpP proteins was determined by western blot assays using antibodies against CrpP from plasmid pUM505. Three *crpP* homologue genes were cloned and transferred to *Escherichia coli* J53-3 as recipient strain.

Results: The *crpP* gene was identified in four (5.19%) ESBL-producing isolates and five (7.35%) ESBL-producing transconjugants with plasmids from clinical isolates. Analysis of the deduced amino acid (aa) sequence of the CrpP protein homologues revealed that they all corresponded to small proteins (63–70 aa) with an identity of 10.1%–43.7% with respect to the pUM505 CrpP sequence. In addition, all *crpP*-positive transconjugants expressed a CrpP protein. Finally, transfer of *crpP* homologues conferred lower ciprofloxacin susceptibility to *E. coli*.

Conclusions: These findings indicate the presence of *crpP* genes among ESBL-producing isolates from Mexican hospitals and point to widespread *crpP*-type genes in old Enterobacteriaceae clinical isolates (from 1994). CrpP probably confers resistance by means of the phosphorylation of ciprofloxacin.

Introduction

Ciprofloxacin, an antibiotic of the fluoroquinolone family, is the most active quinolone against *Pseudomonas aeruginosa*. This antibiotic is widely used to treat infections caused by this bacterium.¹ In addition, ciprofloxacin is used to treat several Gram-negative and Gram-positive bacterial infections, and is the most commonly prescribed antibacterial drug to date.² The most serious infections caused by Gram-negative bacteria occur in clinical settings and are commonly caused by enterobacteria (mostly *Klebsiella pneumoniae*), *Acinetobacter* and *P. aeruginosa*.^{3,4} The emergence and dissemination of resistant bacteria have been shown to be directly related to antibiotic consumption.⁴ In addition, it has been

reported that horizontal transfer of plasmids containing antibiotic resistance genes is an essential mechanism for the dissemination of antimicrobial resistance.⁵ There are three mechanisms of resistance to quinolones: (i) reducing quinolone accumulation through changes in expression of efflux pumps or porin channels; (ii) altering or protecting the antibiotic targets involving mutations in genes encoding target enzymes; and (iii) enzymatic antibiotic modification mediated by plasmids.^{6–8} The first enzymatic mechanism described as conferring resistance to ciprofloxacin and norfloxacin is mediated by the AAC(6′)-Ib-cr enzyme through a variant of the gene encoding aminoglycoside acetyltransferase AAC(6′)-Ib.⁷

Recently, our group reported that the CrpP enzyme, encoded by the pUM505 plasmid, isolated from a *P. aeruginosa* clinical isolate, confers decreased susceptibility to ciprofloxacin when transferred to the *Escherichia coli* J53-3 strain.⁸ CrpP possesses ATP-dependent phosphotransferase activity for ciprofloxacin and it is suggested that when ciprofloxacin is phosphorylated the antibiotic is degraded.⁸

The aim of this study was to analyse the presence of CrpP homologues in a collection of clinical isolates of ESBL-producing Enterobacteriaceae and in plasmids containing ESBLs of nosocomial origin, in an initial attempt to quantify the spread of the *crpP* gene and to understand the factors that favour the prevalence of this gene in bacteria from hospital settings.

Materials and methods

Bacterial strains and culture media

Seventy-seven ESBL-producing clinical isolates of Enterobacteriaceae and 68 transconjugants of the *E. coli* J53-2 strain containing plasmids encoding ESBLs obtained from clinical isolates from hospitals in Mexico between 1988 and 2012 were included in this study (Tables S1 and S2, available as [Supplementary data](#) at JAC Online). The clinical isolates and transconjugants are part of the collection of the Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública (CISEI/INSP), Cuernavaca, Mexico. The clinical isolates were previously characterized by their resistance to multiple antibiotics,⁹ and they included the following species: *E. coli* (44); *K. pneumoniae* (25); *Enterobacter cloacae* (4); *Enterobacter aerogenes* (1); *Enterobacter agglomerans* (1); *Klebsiella cryocrescens* (1); and *Serratia* spp. (1). Transconjugants contained at least one plasmid harbouring an ESBL enzyme transferred from *E. coli* (34), *K. pneumoniae* (33) and *Klebsiella oxytoca* (1) clinical isolates.

E. coli JM101¹⁰ and J53-3 (F⁻, met⁻, pro⁻, Rif^R)¹¹ were utilized as recipient strains for plasmids or in antibiotic susceptibility tests. *E. coli* J53-2 (pUC_ *crpP*) and *E. coli* J53-3 (pUC_ *crpP*)⁸ were included as a positive control for PCR assays and to evaluate quinolone susceptibility, respectively. Nutrient broth and LB broth¹² were used as culture media (for solid media, 1.5% agar was added), and were supplemented with ampicillin (100 mg/L) when necessary.

Identification of *crpP* gene homologues

Hybridization assays

ESBL-producing clinical isolates were inoculated into LB broth and, after overnight growth at 37°C, total DNA was obtained by heat shock, boiling two or three colonies resuspended in 100 µL of distilled water for 5 min. Samples were cooled on ice for 5 min, boiled again, and centrifuged at 10000 rpm for 2 min. The supernatant was recovered and stored at -20°C until use. The DNA (100–150 ng) contained in 10 µL was placed onto nitrocellulose membranes (Hybond-N+; Amersham) and fixed by UV light exposure. The coding region of the pUM505 *crpP* gene was amplified from *E. coli* J53-3 (pUC_ *crpP*) by PCR using forward oligonucleotide 1D (5'-CGAGCTGCTGTTGCTGCTCTGG-3') and reverse oligonucleotide 1R (5'-CGACCGGTACCGACAAGCTGGAC-3'). To use as a probe for the *crpP* gene, the PCR product was purified with the Wizard SV and PCR Clean-up System (Promega) and labelled with the Gene Images AlkPhos Direct Labelling kit (Amersham). Conditions for labelling, hybridization and signal detection were those recommended by the manufacturer at high stringency (63°C).

PCR assays

Total DNA from transconjugants with plasmids from ESBL-producing clinical isolates was obtained as described above. This DNA was used to identify

crpP gene homologues by PCR using oligonucleotides 1D and 1R. DNA from *E. coli* J53-2 (pUCP20) and *E. coli* J53-2 (pUC_ *crpP*) was used as a negative and positive control, respectively. PCR was performed as follows: 5 min at 95°C (1 cycle), 30 s at 95°C, 30 s at 64°C, 30 s at 72°C (35 cycles) and 5 min at 72°C. PCR products were visualized after electrophoresis in 0.7% agarose gels in TAE buffer.¹²

Bacterial growth and susceptibility tests

Bacteria were routinely grown by diluting 1:50 overnight cultures in tubes with 4 mL of fresh medium. After incubating for 18 h at 37°C under constant shaking, growth was monitored as OD at 590 nm with a spectrophotometer (Spectronic 21, Milton Roy).

Antimicrobial susceptibility of all isolates and transconjugants was initially determined with the MicroScan (Dade) system, using the Combo 14 panel. Subsequently, MICs of ciprofloxacin for *E. coli* J53-3 transconjugants with *crpP* gene homologues were determined by microdilution in Mueller-Hinton broth following CLSI recommendations.¹³ *E. coli* ATCC 25922 was used as a reference strain.

Plasmid isolation and conjugation experiments

Matings were performed as described by Miller,¹⁴ using *E. coli* J53-3 (F⁻, Rif^R, NAL^S), a more-susceptible *E. coli* bacterial background, as recipient strain. Transconjugants were selected on Luria agar plates supplemented with rifampicin (100 mg/L) and cefotaxime (1 mg/L) and were designated TT2982, TT1235, TT2424, TT2918 and TT5816. DNA was extracted from *crpP*-positive transconjugants from *E. coli* J53-3 containing plasmids encoding ESBL genes, according to the method described by Kieser.¹⁵ DNA was visualized after electrophoresis in 1% agarose gels stained with ethidium bromide.

Production and purification of antibodies directed against CrpP

Polyclonal antibodies were produced using the CrpP protein purified as described by Chávez-Jacobo *et al.*⁸ Two rabbits were inoculated utilizing the following inoculation scheme. On day 1, 1 mL of rabbit blood was collected to obtain pre-immune serum, after which the rabbits were inoculated with 500 µg of CrpP protein. After 3 weeks, the rabbits were inoculated with a second dose of CrpP protein. Finally, after 6 weeks, the rabbits were bled and serum was collected (immune serum). The antibodies were then purified by precipitation with 40% ammonium sulphate.¹²

Western blot analysis

Overnight cultures of *E. coli* J53-3 transconjugants with plasmids from ESBL-producing clinical isolates were disrupted by sonication to clarity, and debris was removed by centrifugation at 15000 rpm in a Dynamica Velocity 14R centrifuge. Protein quantification of the samples was performed by the Lowry method. Total proteins (100 µg) from the cell lysates were boiled at 95°C in buffer (50 mM NaH₂PO₄ pH 7.5, 300 mM NaCl, 10 mM EDTA) and separated using 14% SDS-PAGE. Then, the proteins were electrotransferred onto nitrocellulose membranes (Hybond-ECL; Amersham, Arlington Heights, IL, USA). The membranes were blocked for at least 1 h at room temperature in Tris-buffered saline-0.1% Tween-20 (TBS-T) containing 5% non-fat dry milk and then incubated with TBS-T containing the anti-CrpP primary antibody at the dilutions indicated. After three washes of 15 min each in TBS-T, the membranes were incubated with peroxidase-conjugated anti-rabbit antibodies for 1 h. After three washes of 15 min with TBS-T, ECL (Amersham) detection was performed and hybridization on the membranes was determined using a ChemiDoc MP Imaging System (Bio-Rad, CA, USA).

Genetic techniques and sequence analysis

General molecular genetics techniques were employed according to standard protocols.¹² Sequencing of *crpP* gene homologues was carried out at Elim Biopharmaceuticals, Inc. (Hayward, CA, USA). To obtain the *crpP* gene sequences, a partial sequence of each *crpP* gene was first obtained using oligonucleotides 1D and 1R (which amplify an internal region) and total DNA from each transconjugant as PCR template. Once the partial DNA sequences had been obtained, they were used to design a pair of oligonucleotides for each *crpP* gene to allow a second DNA sequencing by inverse amplification using the total DNA from each transconjugant as template. Accession numbers (GenBank) of the *crpP* genes from X1235, X2424, X2918, X2982 and X5816 transconjugants are MK093947, MK093948, MK093949 MK093950 and MK093951, respectively. Protein sequence alignments were calculated with the ClustalW2 package (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Potential promoter sequences were searched for using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Secondary protein structure was determined using the MINNOU (Membrane protein Identification withOUT explicit use of hydrophathy profiles and alignments) program (<http://minnou.cchmc.org/>). The recombinant constructions were verified by DNA sequencing carried out at Elim Biopharmaceuticals.

Cloning of *crpP* gene homologues

crpP gene homologues were obtained by PCR using plasmid DNA from TT2424, TT2918 and TT2982 *E. coli* J53-3 transconjugants. The *crpP* gene from TT2424 (297 bp) was amplified using oligonucleotides TT-2424-DIR (5'-GGTGGGAGCACTGGATCCAACCTAG-3'; forward) and TT-2424-REV (5'-GTGCAAGCTTGGCGGCCATTA-3'; reverse) designed with BamHI and HindIII endonuclease sites (underlined), respectively. The *crpP* gene from TT2918 (286 bp) was amplified using oligonucleotides TT-2918-DIR (5'-GAGAAGAAGGATCCAGCACCT-3'; forward) and TT-2918-REV (5'-CTCCTTCAACCAAGCTTCAAGTAA-3'; reverse) designed with BamHI and HindIII endonuclease sites (underlined), respectively. The *crpP* gene from TT2982 (286 bp) was amplified using oligonucleotides TT-2982-DIR (5'-TGGGATCCGTACTTCAAGCCG-3'; forward) and TT-2982-REV (5'-CAAGTGAAGCTTCCCTTC-3'; reverse) designed with BamHI and HindIII endonuclease sites (underlined), respectively. PCR fragments were purified as described previously and were cloned into the pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA), and recombinant plasmids were transferred into the JM101 *E. coli* strain by electroporation. Transformants were selected on LB agar plates with ampicillin. To subclone *crpP* gene homologues into the pUCP20 shuttle vector, recombinant plasmids from pJET derivatives were digested with restriction enzymes BamHI and HindIII, and DNA fragments were ligated into the corresponding sites of pUCP20.¹⁶ Recombinant plasmids were transferred by electroporation to *E. coli* J53-3 and the transformants were selected by culturing on LB agar with ampicillin.

Results

Identification of *crpP*-homologue genes

To investigate the distribution of the *crpP* gene in clinical isolates, a collection of 77 ESBL-producing clinical isolates of enterobacteria causing nosocomial infections in Mexico was used (Table S1). *crpP* homologues from the ESBL-producing isolates were assayed by dot-blot hybridization using a PCR probe of the *crpP* gene obtained from the pUC_ *crpP* plasmid. The results revealed a positive hybridization signal in four clinical isolates (10-221, 04-242, 01-1288, 01-1393) obtained during the years 2008–11 (Figure S1a and Table S1), indicating the possession of a *crpP* homologue.

The presence of *crpP* homologues in these isolates was confirmed by PCR amplification using oligonucleotides 1D and 1R. The

results showed a band of the size expected (177 bp) for the *crpP* gene from pUM505 when DNA from *E. coli* J53-2 (pUC_ *crpP*) was used as a template but not when DNA from *E. coli* J53-2 (pUCP20) was used. In addition, the four isolates identified displayed a sharp DNA band of ~200 bp, corresponding to *crpP* (Figure S1b), confirming that *crpP* homologues are present in the clinical isolates and indicating that this gene is present in bacteria from hospitals. Ciprofloxacin susceptibility of *crpP*-positive isolates as determined by MicroScan showed that three (10-221, 04-242 and 01-1288) were resistant to this antibiotic (Table S1). These data suggest that *crpP* homologues could be responsible for changes in ciprofloxacin susceptibility.

With the purpose of determining whether *crpP* gene homologues might be located on mobile elements, a collection of 68 ESBL-producing transconjugants with plasmids from clinical isolates from hospitals in Mexico was employed. Identification of *crpP* homologues was performed by PCR employing oligonucleotides 1D and 1R. The results showed a band of the size expected (177 bp) for the *crpP* gene from pUM505 when DNA from *E. coli* J53-2 (pUC_ *crpP*) was used as a template but not when DNA from *E. coli* J53-2 (pUCP20) was used. In addition, the results showed that five of the transconjugants (X2982, X1235, X2424, X2918 and X5816) displayed a sharp DNA band of ~200 bp, corresponding to *crpP* (Figure S2), indicating that *crpP* genes are present in plasmids from clinical isolates. Additionally, ciprofloxacin susceptibility MicroScan tests of the *crpP*-positive transconjugants demonstrated that all were resistant to the antibiotic (Table S2). These results indicate that the *crpP* gene is present in clinical isolates on conjugative plasmids, suggesting that spread of this gene to other bacteria in the clinical environment is possible.

Sequence analysis of *crpP* homologues

In silico analysis of the nucleotide sequence of *crpP* homologues from plasmids of ESBL-producing isolates revealed a putative conserved promoter region of the σ^{70} -type (Figure 1a) at the 5' end, similar to that of the *crpP* gene from the pUM505 plasmid, suggesting that these genes are functional and that they have similar regulation. The proteins encoded by these *crpP* homologues were all small, with sizes ranging between 63 and 70 aa, similar to the length of the pUM505 CrpP protein of 65 aa (Figure 1b). With the exception of the CrpP sequence encoded by the transconjugant X2918, which exhibited 10.1% identity and 18.8% similarity with the pUM505 CrpP protein, the other CrpP sequences showed 33.3%–43.7% identity and 43.8%–53.5% similarity (Table 1), suggesting that these putative proteins represent CrpP homologues and that they probably confer decreased susceptibility to ciprofloxacin by the same mechanism as that of the pUM505 CrpP. The CrpP proteins are more conserved in the N-terminal region, displaying 40%–65.7% identity with the first 35 aa of CrpP from pUM505, with the exception of the CrpP protein from X2918, which exhibited 17.6% identity. However, the C-terminal region of the CrpP proteins displayed a low identity (5.7%–10.5%) with the last 30 aa of CrpP from pUM505, suggesting that the N-terminal region could possess residues important for their function. In addition, it was found that all of the putative CrpP homologues possessed the Gly residue at position 7, which has been previously suggested to be involved in protein function.⁸ Residue Ile at position 27 of CrpP from pUM505, which was also

suggested to be important for ATP binding,⁸ was conserved in three of five of the CrpP homologues (X2982, X1235 and X5816); the CrpP protein from X2424 showed a change to leucine at position 27, suggesting that the essential residue could be changed by mutation for a similar aa (Figure 1b). Additionally, the alignment shows that the residues Thr8, Asp9, Lys33, Gly34 and Cys40 are conserved in all the proteins, suggesting that these residues could have a role in CrpP activity. However, an extended analysis of the role of these residues in CrpP enzymatic function is required. To explore whether the CrpP proteins possess motifs that could be related to its function, a secondary-structure analysis was conducted. The results revealed that the pUM505 CrpP protein possesses a predicted secondary structure composed of three α helices (α 1 from residues 21–25, α 2 from residues 28–32 and α 3 from residues 46–63) (Figure 1c). This structure was not conserved in the CrpP homologues; however, proteins from transconjugants X2982, X2424 and X5816 exhibited a similar α 1 helix and part of the α 3 helix, whereas X2424, in addition to α 1 and α 3 helices, possessed the α 2 helix. These analyses suggest that CrpP homologues do not possess a conserved secondary structure; however, it could mean that conserved residues may play an important role in CrpP function.

Determination of CrpP protein homologue expression

With the purpose of determining whether *crpP* gene homologues from ESBL-producing isolates produce CrpP-type proteins, plasmids from *crpP*-positive transconjugants were transferred to the *E. coli* J53-3 strain and western blot assays were performed using a polyclonal antibody against pUM505 CrpP. The results showed only one band corresponding to the CrpP protein in the transconjugants tested (Figure 2). These results clearly indicate that the *crpP* genes are expressed in the clinical isolates and their products are recognized by the antibody against CrpP protein, suggesting similar conformation features among them.

Determination of CrpP protein homologue functionality

In order to demonstrate the functionality of CrpP homologues from the plasmids of ESBL-producing isolates, the *crpP* genes from the TT2424, TT2918 and TT2982 transconjugants were cloned into the pUCP20 vector and transferred to *E. coli* J53-3. The results showed that the three *crpP* genes were able to increase the MICs of ciprofloxacin in *E. coli* J53-3 (Table 2). Transformants with the *crpP* gene from TT2424 and TT2918 exhibited similar MICs to those conferred by pUM505 *crpP*, whereas *crpP* transformants from TT2982 increased the MIC 2-fold with respect to the pUM505 *crpP* gene (Table 2). Furthermore, as previously described, the transconjugants that encode the CrpP-homologous proteins showed resistance to ciprofloxacin (Table S2), suggesting that the plasmids contained in these strains, in addition to possessing *crpP*, contain other genes that encode additional quinolone-resistance mechanisms. These results indicate that plasmid-borne *crpP* genes are spread among clinical isolates and suggest that they could participate in decreasing susceptibility to ciprofloxacin, most likely through the phosphorylation of the antibiotic, a mechanism described for the CrpP protein of the pUM505 plasmid, together with other mechanisms of plasmid-encoded resistance.

Discussion

Quinolones have been extensively used to treat a large number of bacterial infections and ciprofloxacin is the most active antibiotic of this group, utilized to treat infections generated by *P. aeruginosa*.^{1,17} However, it is known that quinolone resistance in infecting microorganisms can be associated with therapeutic failure.¹⁸ In addition to the quinolone resistance mediated by chromosomal gene mutations (*gyrA*, *parC*), resistance can be attributed to plasmid-mediated quinolone resistance (PMQR) genes such as *qnr*, *qepA*, *oqxAB*, *aac(6')-Ib-cr* and *crpP*.^{6–8} Several studies have been conducted to identify the presence of PMQR genes, as well as *qnr* and *aac(6')-Ib-cr*, in enterobacterial isolates from humans, animals and food, and the results showed that the average prevalences of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* in a compiled database were 1.5%, 4.6%, 2.4% and 10.8%, respectively.^{19–22}

The *crpP* gene, obtained from the pUM505 plasmid isolated from a *P. aeruginosa* clinical isolate, was identified in 1986 and fully sequenced in 2011.^{23,24} Recently, our group reported that the *crpP* gene encodes a novel mechanism that decreases susceptibility to ciprofloxacin by means of the enzymatic phosphorylation of the antibiotic;⁸ however, there are no studies describing the presence of this gene in clinical isolates. To investigate whether this gene could be spreading in other groups of Gram-negative bacteria, a collection of ESBL-producing enterobacterial clinical isolates and transconjugants containing plasmids encoding ESBL genes was analysed. *crpP* gene homologues were identified in these isolates with a frequency of 5.19%, indicating that this gene is moderately spread in bacteria causing infections in hospitals. In general, the prevalence of the *crpP* gene was higher among *E. coli* isolates (75%), followed by those of *K. pneumoniae* (25%). Comparing the prevalence of other PMQR elements reported in previous studies⁹ with those found in the clinical isolates studied here, we found the *crpP* gene at a lower frequency than the *qnr* (21%) and *aac(6')-Ib-cr* (43%) genes. The reduced susceptibility to ciprofloxacin of these *crpP*-positive isolates suggests that the *crpP* gene could play a role in susceptibility to this antibiotic.

Because quinolone resistance usually resides on plasmids, with the purpose of determining whether *crpP* gene homologues could be located on mobile elements, we also analysed a collection of 68 transconjugants with plasmid-borne ESBL genes obtained from clinical isolates from hospitals in Mexico. The results showed that 7.35% of the transconjugants possess a *crpP* gene, indicating that the *crpP* gene is moderately spread in plasmids from clinical isolates, and suggest that this gene could be mobilized by horizontal gene transfer and contribute to the prevalence of clinical bacteria with decreased susceptibility to ciprofloxacin.

crpP homologues displayed a putative σ^E -type conserved promoter, suggesting similar regulation. σ^E is the *P. aeruginosa* orthologue of *E. coli* and *Salmonella* σ^E , an alternative sigma factor that directs the transcription of genes in response to extreme stress conditions;²⁵ however, more analysis of their effect on *crpP* genes is required. In general, *crpP* genes encode small proteins, similar in size to CrpP from pUM505, and are more conserved in the N-terminal region than in the C-terminal region, with low aa sequence identity to CrpP of pUM505. This suggests that CrpP-type proteins could be a group of proteins not conserved in primary structure but possessing the same function. It has been reported that the aminoglycoside O-phosphotransferases (APH), a family of

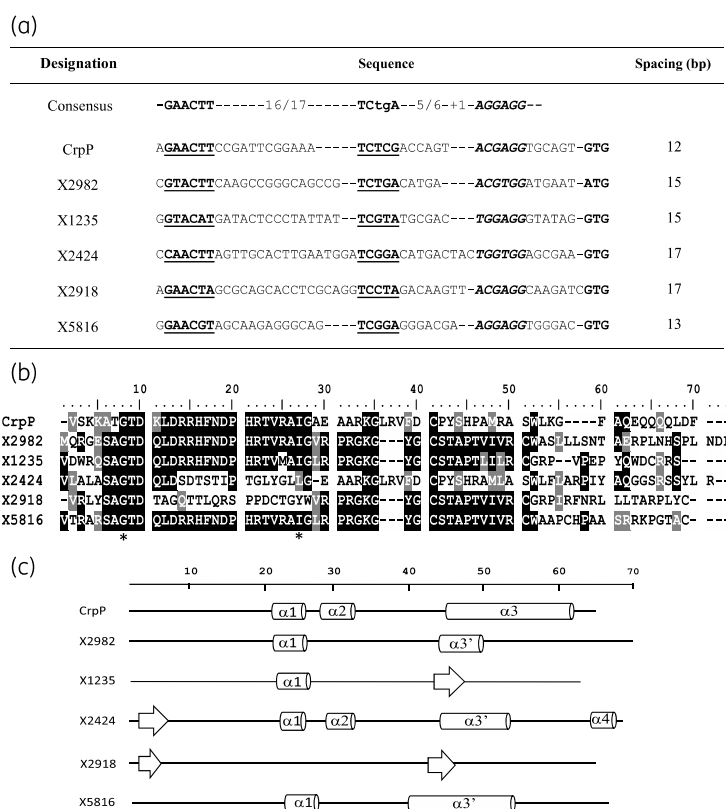


Figure 1. Analysis of regulatory region and comparison of products from *crpP* homologue genes. (a) Alignment of the putative AlgU promoters and the consensus AlgU (σ^E)-type promoter. Conserved nucleotides are shown in capital letters, while less-conserved nucleotides are shown in lowercase. The -10 and -35 regions of the consensus sequence are indicated in bold and are underlined. The putative RBS binding site is indicated in italics, and the start codon is highlighted in bold. Nucleotides conserved in promoters from *crpP*-homologue genes are underlined. (b) Multiple sequence alignment of CrpP-homologue proteins. Alignments include sequences of CrpP from pUM505 and putative CrpP-homologue proteins encoded by plasmid isolates from ESBL-producing transconjugants. Filled squares indicate conserved catalytic residues identified in the CrpP proteins. Asterisks denote putative conserved catalytic residues in the CrpP protein from pUM505. (c) Prediction of secondary-structure elements using the MINNOU program according to CrpP proteins from pUM505 and transconjugants are shown. The line at the top shows the numbers of aa of the proteins. Barrels correspond to α helices and arrows to β sheets.

Table 1. Similarity and identity of the deduced aa sequences of CrpP homologue proteins versus CrpP from pUM505

		Identity/similarity (%)				
	CrpP	X2982	X1235	X2424	X2918	X5816
CrpP	100/100	34.2/43.8	33.3/46.4	43.7/53.5	10.1/18.8	34.2/47.1
X2982		100/100	57.1/67.1	24.7/34.2	37.1/41.4	65.7/72.9
X1235			100/100	26.8/32.4	37.9/43.9	63.6/71.2
X2424				100/100	14.1/22.5	22.5/35.2
X2918					100/100	39.4/42.4
X5816						100/100

enzymes that confer resistance to aminoglycosides, share low sequence identity (20%–40%) but contain important conserved motifs, particularly at the C-terminal region, which is primarily responsible for catalysis and aminoglycoside binding, whereas the ATP-binding domain is mainly located in the N-terminal region.²⁶ Also, the sequences of the catalytic and ATP-binding domains vary between individual APH enzymes and among APH subfamilies.²⁶ The CrpP proteins show differences in their secondary structure, but several aa residues present in CrpP from pUM505 are conserved in all of the CrpP homologues, as well as Gly7, Thr8, Asp9, Lys33, Gly34 (located at the N-terminal region) and Cys40, suggesting that these residues could be important in the protein's function. Thus, the CrpP proteins may confer resistance to ciprofloxacin,

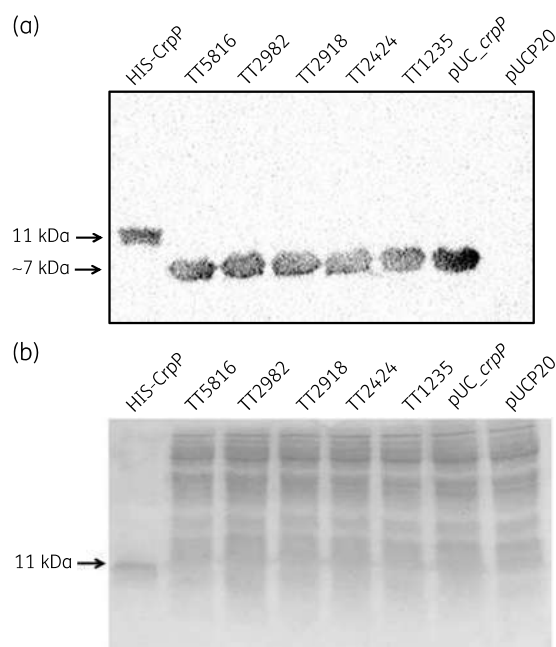


Figure 2. Expression of CrpP protein homologues from transconjugant ESBL-producing isolates. (a) Autoradiography of the expression of CrpP-homologous proteins from transconjugants of *E. coli* J53-3 with plasmids encoding ESBLs. An antibody against CrpP was utilized for the western blot analysis. Total protein (250 µg) from extracts of cultures of the indicated transconjugants and 0.05 µg of His-CrpP purified protein and *E. coli* J53-3 (pUC_crpP) were used. The J53-3 (pUCP20) strain was included as a negative control. (b) Ponceau red staining of the western blot membrane for CrpP protein detection. Data are reported as representative results from two independent experiments.

Table 2. Ciprofloxacin susceptibility tests

<i>E. coli</i> strain	Ciprofloxacin MIC (mg/L)
ATCC 25922	0.004
J53-3	0.004
J53-3 (pUC_crpP)	0.06
J53-3 (pUC_crpP2424)	0.06
J53-3 (pUC_crpP2918)	0.06
J53-3 (pUC_crpP2982)	0.125

CLSI MIC breakpoints for ciprofloxacin are ≤1.0 mg/L (susceptible), 2.0 mg/L (intermediate) and ≥4.0 mg/L (resistant).

while variability in the protein sequence may promote the recognition of specific substrates. A deeper study of the participation of the residues conserved among the CrpP proteins could help us understand their role in ciprofloxacin susceptibility.

Expression of CrpP in the transconjugants tested suggests that these proteins could participate in conferring ciprofloxacin resistance. Moreover, transfer of *crpP* homologues from the plasmids of transconjugants conferred on *E. coli* J53-3 a decrease in

susceptibility to ciprofloxacin, indicating that the *crpP* genes encode a functional protein that confers decreased susceptibility to the antibiotic, probably by the mechanism of phosphorylation of ciprofloxacin, similar to that employed by the CrpP protein from pUM505.⁸ Additionally, the ability of the CrpP-homologous protein from TT2918, which showed the lowest identity (10.1%) with CrpP of pUM505, to confer decreased susceptibility to ciprofloxacin in *E. coli* strengthens the idea that conserved residues could be important in the function of CrpP proteins instead of a similar secondary structure. CrpP proteins decrease susceptibility to ciprofloxacin, increasing MIC values with respect to *E. coli* J53-3; however, these changes are not enough to be considered as resistance according to the breakpoint value (≥4 mg/L) reported for this interpretation by the CLSI. Nevertheless, *crpP* is not the first gene described that encodes a mechanism that confers a low level of resistance to ciprofloxacin, in that it has been reported that PMQR genes can confer low-level resistance while facilitating the selection of mutants with a higher level of quinolone resistance.²⁷

Additionally, the transconjugants that encode the CrpP-homologous proteins showed resistance to ciprofloxacin, suggesting that the plasmids contained in these strains contain other genes (in addition to *crpP*) that encode additional quinolone resistance mechanisms, which together could be responsible for the resistant phenotype observed.

In conclusion, our results showed that *crpP* homologues are found in plasmids of enterobacterial isolates of nosocomial origin and suggest that the *crpP* genes may be transferred among hospital bacteria owing to their location within genetic mobile elements, probably co-selected by antibiotic exposure.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 and S2 are available as [Supplementary data](#) at JAC Online.

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Artículos

Plásmidos bacterianos

Karen Cecilia Hernández Ramírez

Sabemos que a diario convivimos con un montón de organismos diminutos llamados bacterias (ver Saber Más, 1:16-18), pero ¿Conocemos qué los ayuda a ser más fuertes y capaces de vivir en todos lados? Las bacterias son microorganismos que se adaptan fácilmente a las distintas condiciones de su ambiente (ver Saber Más 21:10-16) ¿Quién apoya a las bacterias a ser más poderosas?... Parte de la respuesta son "los plásmidos".

¿Qué son los plásmidos?

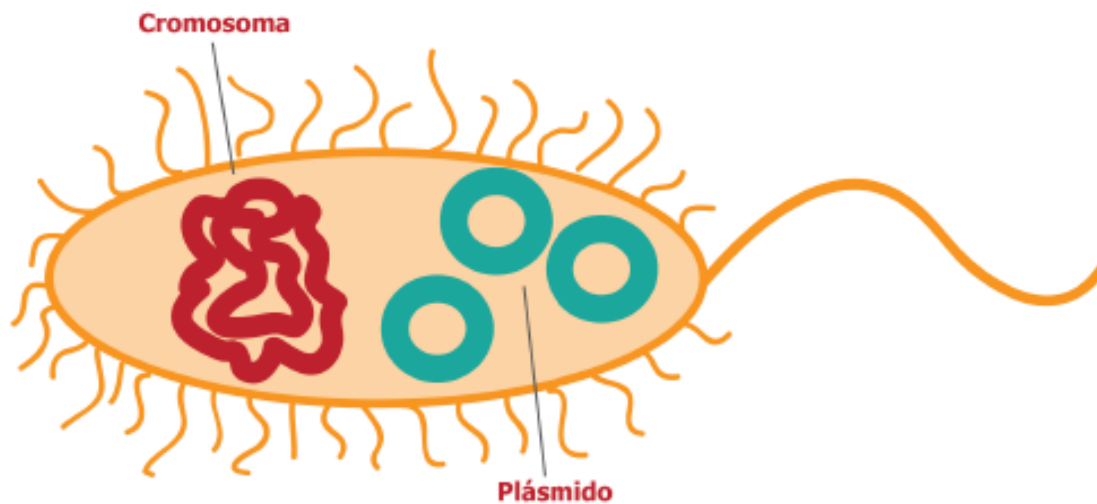
Los plásmidos son moléculas de material genético (ADN) que se replican independientes

del cromosoma bacteriano (ADN que contiene los genes esenciales para la supervivencia de la bacteria).

La palabra plásmido fue dada a conocer por primera vez por el biólogo molecular norteamericano Joshua Lederberg en 1952 (quien obtuvo el premio Nobel de Fisiología y Medicina en 1958). En 1957, durante una epidemia de disentería en Japón, un grupo de investigadores descubrió que ciertas formas bacterianas eran resistentes a los antibióticos empleados para tratar esta enfermedad. Tiempo después se encontró que esta resistencia se debía nada más y nada menos que a los mencionados plásmidos.

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Plásmido Un instrumento genético que facilita a las bacterias adaptarse a su entorno

Sólo tienen lo esencial

Así como todas las personas tenemos características similares que son las que nos definen como humanos, también hay características diferentes que nos hacen distintos entre sí; por su parte los plásmidos poseen propiedades semejantes que están presentes en todos ellos, pero también contienen características que los hace ser especiales. Algunas de estas características son las siguientes:

- La mayoría son muy pequeñitos en comparación con el cromosoma bacteriano. Esto lo podemos apreciar mejor en la siguiente figura y, aunque casi todos ellos son circulares, hay algunos lineales.
- Cada plásmido contiene al menos una secuencia de ADN que sirve como un origen de replicación lo cual permite al ADN ser reproducido en forma independientemente del cromosoma.
- Poseen genes que dan ventajas a las bacterias que los contienen, de lo que hablaremos más adelante.

Los hay de todo tipo

Los plásmidos han sido encontrados en casi todas las bacterias y Arqueas, además de

que también pueden estar presentes en levaduras (hongos microscópicos) (ver *Saber Más*, 13:7-9). Por lo tanto, si se han encontrado en una cantidad enorme de bacterias, se conocen muchísimos plásmidos diferentes también. Por ejemplo, de *Escherichia coli* se han aislado más de 300 plásmidos.

Debido a su gran número en la naturaleza, una de las clasificaciones que se les ha dado es de acuerdo al tipo de genes que contiene:

- Los plásmidos de resistencia son aquellos que le permiten a la bacteria hacerle frente a un veneno, un antibiótico, metales pesados tóxicos, etc.
- Los plásmidos degradativos ayudan a la célula bacteriana a digerir sustancias que no son habituales en su entorno. Por ejemplo, *Burkholderia xenovorans* tiene un plásmido que le auxilia a degradar compuestos sintéticos contaminantes como los compuestos policlorados.
- También existen plásmidos que producen toxinas (sustancias tóxicas).
- Y también están los plásmidos de virulencia, que son la causa de que una bacteria tenga mayor capacidad para causar una enfermedad.

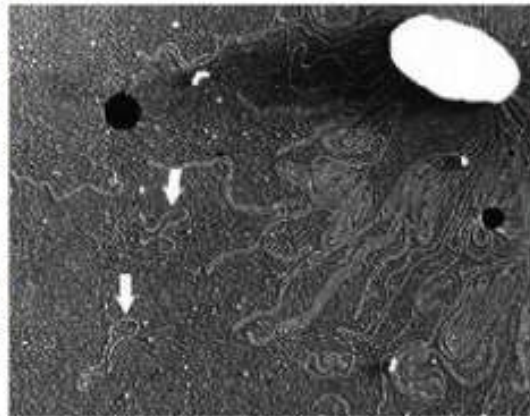
Los grandes aliados de las bacterias

Los plásmidos, en general, no contienen información esencial para el crecimiento de las bacterias, entonces ¿Cómo pueden ser sus aliados?

Como ya se había mencionado, los plásmidos rara vez contienen genes necesarios para el crecimiento bacteriano, ya que los genes esenciales se encuentran en el cromosoma; sin embargo, estos elementos les dan ventajas a las bacterias en condiciones de crecimiento determinadas.

El ejemplo más común es el de los plásmidos que contienen genes de resistencia a un determinado antibiótico, de manera que el plásmido únicamente supondrá una ventaja en presencia de ese antibiótico. La resistencia a los antibióticos se ha encontrado en bacterias patógenas causantes de enfermedades como: tifoidea, meningitis, gonorrea y otras.

Los plásmidos actúan proporcionando la información necesaria para modificar o destruir el antibiótico para que éste no dañe a la bacteria. La resistencia a los antibióticos hoy en día representa un serio problema de salud pública a nivel mundial (ver Saber Más, 14:4-5). Como podemos ver, los plásmidos definitivamente son esos compañeros que siempre están con las bacterias para ayudarlas en su entorno.



En esta fotografía la célula bacteriana (óvalo blanco arriba a la derecha) se rompió con cuidado, de manera que el ADN ha quedado intacto. Los plásmidos (señalados con flechas) son las estructuras circulares mucho más pequeñas que el ADN cromosómico (ovillo de la derecha).

¿En qué nos pueden ayudar los plásmidos?

Al parecer, los plásmidos benefician a las bacterias, pero ¿Será que también nos puedan ayudar a nosotros?

Aunque parezca un poco ilógico ¡Sí pueden!

Sólo daré dos ejemplos. Los plásmidos tienen aplicación en ingeniería genética, a la fecha, se han construido incontables plásmidos artificiales en los laboratorios de biología molecular o de ingeniería genética llamados vectores, que son fáciles de manipular y a los que se les pueden introducir nuevos genes de nuestro interés. Un ejemplo muy importante es la insulina que es empleada para el tratamiento de la diabetes, éste fue el primer caso de una proteína producida por ingeniería genética, aprobada para uso en humanos desde 1982. De esta forma, los plásmidos pueden ser auxiliares en el control de enfermedades. Por otro lado, en 1999, investigadores reportaron que un plásmido bacteriano contiene los genes necesarios para la degradación de naftaleno, bifenilo y tolueno que son contaminantes del ambiente. Por lo tanto, los plásmidos también pueden ser usados en biorremediación, una tecnología dirigida a proteger nuestro planeta.

SaberMás

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