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"Estudio del mecanismo de regulación transcripcional por la proteína RhlR de *Pseudomonas aeruginosa*".

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BIOL. GERARDO ENRIQUE MEDINA BASULTO

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Indice

Indice de tablas

Indice de figuras

Introducción

Resultados I

Resultados II

Resultados III

Resultados y discusión adicionales

PAGINACIÓN DISCONTINUA

I. Resumen

Pseudomonas aeruginosa es una bacteria gram negativa a menudo asociada con infecciones en pacientes inmunocomprometidos, con fibrosis quística o quemaduras graves. La base de su patogenicidad es la producción y secreción de factores de virulencia tales como proteasas, hemolisinas, exotoxinas y pigmentos capaces de provocar daños en tejidos humanos y de otros mamíferos. La regulación de los genes que codifican para factores de virulencia, depende principalmente de un sistema de comunicación entre células llamado detector de quórum. Como parte de los factores de virulencia secretados por *P. aeruginosa*, se encuentran los ramnolípidos, que son moléculas anfipáticas con propiedades hemolíticas y surfactantes, siendo esta última propiedad importante por su potencial aplicación en la industria y en biorremediación.

El sistema detector de quórum que regula la producción de ramnolípidos en *P. aeruginosa*, consta de una molécula sintetizada por la proteína RhlI llamada *N*-butanoil-L-homoserin lactona (BHL) que se libera al medio; y el regulador transcripcional RhlR. El complejo RhlR-BHL activa los genes *rhlAB*, transcritos como un operón y que codifican para la ramnosil transferasa 1 que sintetiza el monoramnolípido (L-ramnosil-β–hidroxidecanoil-β-hidroxidecanoato).

En el presente trabajo se presenta la caracterización de los promotores de los genes *rhlA* y *rhlR*. Utilizando fusiones *prhlA-lacZ* se encontró que *rhlA* comienza a transcribirse en fase pre-estacionaria, aun si tanto BHL como RhlR están presentes desde el principio del cultivo. También se determinó el inicio transcripcional de *rhlA* en una cepa σ^{54-} de *E. coli*, encontrando que el promotor no depende de dicho factor sigma contrariamente a lo que se había propuesto. Por medio de ensayos de "footprinting" *in vivo*, se determinó que el regulador RhlR se une a la región reguladora de *rhlA* tanto en presencia como en ausencia de autoinductor, siendo en esta última condición un regulador negativo de la transcripción de *rhlA*. Por medio de fusiones *prhlR-lacZ* se encontró que la expresión de *rhlR* depende de los reguladores σ^{54}, LasR y VFR, los cuales además se observó que afectan la expresión de uno o más de los inicios transcripcionales. Se determinó también que la expresión de *rhlR* y la producción de ramnolípidos, son influenciadas fuertemente por el pH, la relación carbono / nitrógeno, la fuente de carbono utilizada y la concentración de fosfatos en el medio, lo cual pone de manifiesto que el regulón *rhl* no sólo responde a densidad celular sino que es un importante modulador de la respuesta detectora de quórum a distintos estímulos ambientales.

II. Abstract

Pseudomonas aeruginosa is a gram-negative bacterium often associated with infections of immunocompromised patients, cystic fibrosis or severe burn wounds. The basis of its pathogenicity is the production and secretion of virulence factors such as proteases, haemolysins, exotoxins and pigments capables of causing tissue damage in humans and other mammals. Regulation of the genes encoding virulence factors is primarily dependent upon an intercellular communication system called quorum-sensing. Among virulence factors secreted by *P. aeruginosa* are rhamnolipids, which are amphipatic molecules with haemolytic and surfactant properties that have potential applications in industry and bioremediation.

The quorum-sensing system that regulates rhamnolipids production in *P. aeruginosa,* consists of a pair of proteins: the RhlI protein that catalyzes the formation of the so-called autoinducer N-butanoyl-L-homoserine lactone (BHL), and the transcriptional regulator RhlR. The RhlR-BHL complex activates the *rhlAB* genes, which are transcribed as an operon and encode rhamnosyltransferase 1 that directs the synthesis of mono-rhamnolipid (L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate).

This work presents the characterization of the *rhlA* and *rhlR* promoters. Using *prhlA-lacZ* fusions we found that *rhlA* transcription begins in pre-stationary phase even when RhlR and autoinducer are present from the beginning of the culture. It was also determined by "primer extension" assays that the transcription of *rhlA* was not dependent on σ^{54} sigma factor, as reported by other groups. *In vivo* "footprinting" assays determined that RhlR regulator specifically binds to *rhlA* promoter region both, in the presence and absence of the autoinducer. In this last condition RhlR represses *rhlA* transcription.

The results of *prhlR-lacZ* fusions indicated that *rhlR* expression depends on the σ^{54}, LasR and VFR regulators affecting the expression of one or more transcriptional start sites as determined by "primer extension" assays. We also found that *rhlR* expression and rhamnolipids production are influenced by pH, carbon/nitrogen ratio, carbon source and phosphate concentration in the culture medium. These findings point out that the *rhl* regulon not only responds to cellular density but also to environmental factors.

III. Introducción

Pseudomonas aeruginosa es una bacteria gram negativa que crece en el suelo, aguas someras y habitats marinos, así como en aguas residuales, tejidos animales y plantas (Stover et. al., 2000; Hardalo et. al., 1997). Su gran versatilidad radica en la diversidad funcional de su genoma (6.3Mpb), el cual contiene significativamente más familias de genes de distinto origen (grupos parálogos) que otros genomas comparables en tamaño como *E. coli*, *Bacillus subtilis* o *Mycobacterium tuberculosis*. Se cree que en función de tal diversidad génica, *P. aeruginosa* ha emergido como un importante patógeno oportunista de humanos ya que resiste de manera natural a una gran cantidad de antibióticos y desinfectantes que generalmente eliminan a otras bacterias, de tal manera que es un causante común de muerte en pacientes con fibrosis quística, o inmunocomprometidos. *P. aeruginosa* también puede causar bacteremia en pacientes con quemaduras graves, en el tracto urinario cuando se utilizan catéteres o en pacientes que adquieren pneumonia al utilizar respiradores (Bodey, 1983). Por otro lado algunas cepas de *P. aeruginosa* son importantes desde el punto de vista biotecnológico debido a que son capaces de degradar compuestos orgánicos, tales como hidrocarburos alifáticos, compuestos aromáticos y solventes halogenados (Poole, 2001; Maier y Soberón-Chávez, 2000; Wackett, 1998; Hardman, 1991).

Produccion de ramnolípidos por *Pseudomonas aeruginosa*.

P. aeruginosa, crecida bajo condiciones de limitación de nutrientes y a alta densidad celular produce y secreta al medio ramnolípidos, los cuales son glicolípidos que contienen una o dos moléculas de ramnosa unidas por un enlace glicosídico a una o dos moléculas de ácido graso (Jarvis y Johnson, 1949; Hauser y Karnovsky, 1958). En cultivos líquidos, *P. aeruginosa* produce principalmente L-ramnosil-β–hidroxidecanoil-β-hidroxidecanoato (monoramnolípido) y L-ramnosil-L-ramnosil-β–hidroxidecanoil-β-hidroxidecanoato (diramnolípido) (Rendell et. al., 1990).

Los precursores para la síntesis de ramnolípidos provienen de las vías generales de

síntesis de ácidos grasos y del metabolismo central de carbono, así, la parte de ácido graso se separa de la vía general, al nivel de la reducción de grupos cetoacilos por la proteína RhlG (Campos-García et. al., 1998) y la Timidina difosfato-L-ramnosa (TDP-L-ramnosa) se sintetiza a partir de glucosa-1-fosfato por las enzimas codificadas por los genes del operón *rml* (Maier y Soberón-Chávez, 2000; Olvera C., 2000; Rahim et. al., 2000). El gen *algC* codifica para una fosfomanomutasa que cataliza la conversión de manosa-6-fosfato a manosa-1-fosfato necesaria para la síntesis de alginatos, aunque también muestra actividad fosfoglucomutasa participando en la síntesis de glucosa y ramnosa para la producción de lipopolisacáridos (Coyne et. al., 1994). AlgC a través de su actividad fosfoglucomutasa es responsable de la producción de glucosa-1-fosfato necesaria para formar el precursor dTDP-L-ramnosa (Olvera et. al., 1999) **(Figura 1)**.

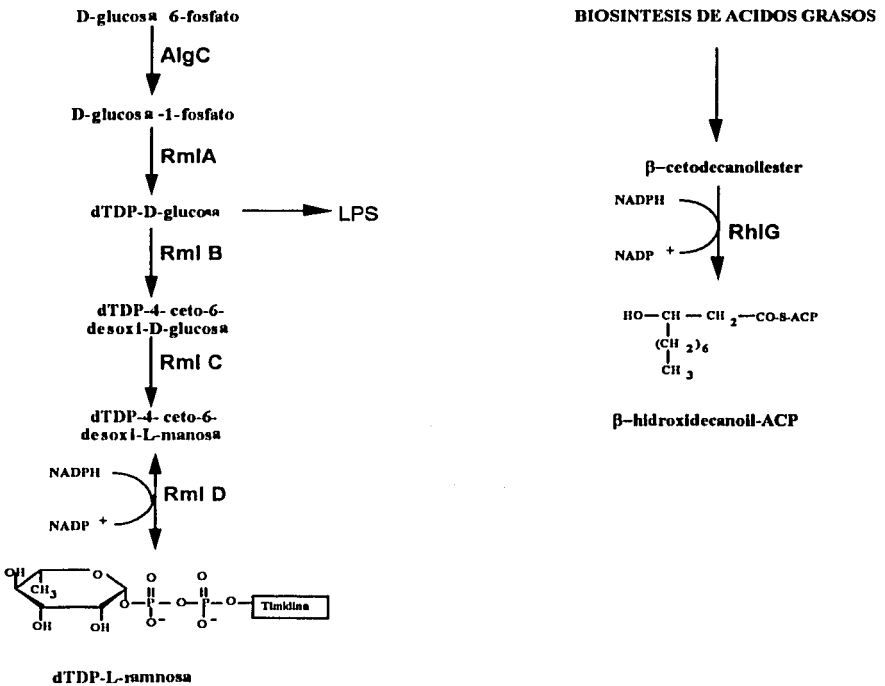

Figura 1. Vía general de la biosíntesis de los precursores dTDP - L - ramnosa y β - hidroxidecanoil - β - hidroxidecanoil - S – CoA, necesarios para la producción de ramnolípidos en *Pseudomonas aeruginosa*.

La biosíntesis de ramnolípidos se lleva a cabo en dos reacciones secuenciales catalizadas por las enzimas ramnosil transferasa 1 y ramnosil transferasa 2 respectivamente, utilizando dTDP-L-ramnosa como donador del grupo ramnosil y β–hidroxidecanoil-β-hidroxidecanoato o monoramnolípido como receptor (Burger et. al., 1963; 1966) (**Figura 2**).

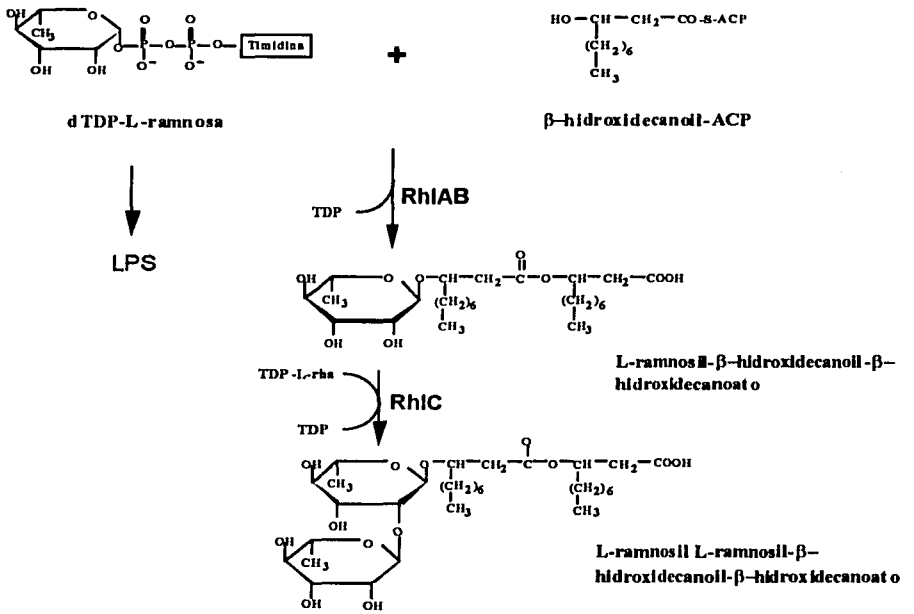

Figura 2. Biosíntesis de ramnolípidos mostrando las reacciones catalizadas por el producto de los genes *rhlAB* y *rhlC* en *Pseudomonas aeruginosa*.

La enzima ramnosil transferasa 1 esta compuesta de dos subunidades codificadas por los genes *rhlA* y *rhlB* siendo RhlA una proteína de membrana interna y RhlB periplásmica y la que posee la actividad catalítica (Ochsner et. al., 1994a). El gen *rhlC* codifica para la ramnosil transferasa 2 (Rahim et. al., 2001), se encuentra en un locus diferente al de *rhlAB* y tiene homología con las ramnosil transferasas necesarias para la síntesis de lipopolisacáridos.

Importancia de los ramnolípidos producidos por *Pseudomonas aeruginosa*

Los surfactantes son moléculas anfipáticas capaces de disminuir la tensión superficial e interfacial entre sustancias de diferente polaridad, los cuales pueden ser de origen biológico o sintético.

Los ramnolípidos son bio-surfactantes con una gran variedad de aplicaciones potenciales tanto en la industria como en bio-rremediación. Así, se ha propuesto su uso en la producción de compuestos químicos de alto valor agregado para el estudio de propiedades superficiales (Ishigami y Suzuki, 1997). Las industrias cosmética y de la salud utilizan grandes cantidades de surfactantes sintéticos para la producción de repelentes, antiácidos, soluciones para lentes de contacto, pastas de dientes, cremas de belleza, antisépticos, etc. Sin embargo los ramnolípidos son rápidamente degradados y poco irritantes por lo que podrían ser utilizados como una alternativa. La adición de ramnolípidos a cultivos puros de *P. aeruginosa*, aumenta su capacidad de utilizar hidrocarburos como única fuente de carbono (Miller, 1995; Zhang and Miller, 1994; 1995), por lo que se esta explorando la posibilidad de utilizarlos en la biorremediación de suelos contaminados por petróleo.

Otra propiedad de los ramnolípidos es su capacidad de adsorber metales pesados como plomo, cadmio y arsénico, por lo que pueden ser utilizados en la biorremediación de sitios contaminados con estos metales (Baath, 1989; Sandrin et. al., 2000). Finalmente los ramnolípidos son altamente efectivos en el control de hongos zoopóricos patógenos de plantas como *Pythium aphanidermatum* y *Phytophthora capsici* (Stanghellini y Miller, 1997), lo que demuestra su versatilidad de usos y la importancia que tiene el lograr su producción a bajo costo para poder utilizarlos de manera amplia.

Regulación transcripcional por el sistema detector de quórum

La gran mayoría de los microorganismos están sujetos a cambios ambientales severos, tales como variación de temperatura, osmolaridad, pH y disponibilidad de nutrientes, por lo que han desarrollado diversos sistemas que les permiten adaptarse a las condiciones cambiantes. Los

sistemas de dos componentes por medio de cascadas de fosforilación, permiten a los microorganismos detectar y responder a factores ambientales con la activación o represión de genes específicos. De igual manera, la expresión de factores sigma alternativos en respuesta a varias señales, permiten especificidad transcripcional a las bacterias, además de las alteraciones en la topología del ADN, que pueden resultar en cambios del patrón de expresión de un microorganismo (Stock et. al., 2000; Wösten, 1998; Perez-Martin y de Lorenzo, 1997; whitehead et. al., 2001).

Otro sistema de detección y respuesta a es la comunicación intercelular por medio de pequeñas moléculas denominadas autoinductores, que son producidas por una población bacteriana hasta alcanzar una concentración crítica, permitiéndoles iniciar una acción concertada por medio de la transcripción de grupos de genes específicos a un mismo tiempo. Dicho sistema ha sido denominado detector de quórum (Fuqua et. al., 1994), y puede utilizar distintos autoinductores, siendo comunes los aminoácidos o péptidos cortos en bacterias gram positivas y los derivados acilados de homoserin lactonas, quinolonas o furanosil borato diester en bacterias gram negativas (Kleerebezem et. al., 1997; Shapiro, 1998; Whitehead, 2001; McKnight et. al., 2000; Chen et.al., 2002). Algunos ejemplos de sistemas detectores de quórum se muestran en la **Tabla 1**.

Tabla 1. Organismos que poseen homólogos de LuxI/LuxR, proteinas regulatorias, autoinductores y funciones reguladas.

Organismo	Fenotipo	Autoinductor sintetasa	Regulador transcripcional	Autoinductor
Agrobacterium tumefaciens	Conjugación	TraI	TraR	OOHL
Chromobacterium violaceum	Antibióticos, exoenzimas, violaceina	CviI	CviR	HHL
Erwinia carotovora	Antibioticos, exoenzimas	CarI	CarR	OHHL
Erwinia stewartii	Exopolisacárido	EsaI	EsaR	OHHL
Escherichia coli	División celular	?	SdiA	?
Vibrio (Photobacterium) fischeri	Bioluminiscencia	LuxI	LuxR	OHHL, HHL
Pseudomonas aeruginosa	Proteasa alcalina, elastasa, exotoxina A, exoenzima S, neuraminidasa, hemolisina.	LasI	LasR	OdDHL
	Quitinasa, elastasa, piocianina, ramnolípidos, RpoS	RhlI	RhlR	BHL
Serratia liquefaciens	Motilidad, fosfolipasa	SwrL	?	BHL

El sistema detector de quórum, fue descrito inicialmente como un regulador de la bioluminiscencia en la bacteria marina *Vibrio fischeri* (Nealson K. H., 1977). Cuando esta bacteria vive de forma planctónica (baja densidad celular) no posee luminiscencia, pero cuando se encuentra viviendo como simbionte en órganos especiales de algunos peces o calamares (donde alcanza alta densidad celular), se induce la producción de luciferasa, otorgando protección a su huésped contra depredadores al eliminar su sombra creada por la luz de la luna (Visick y McFall-Ngai, 2000). El grupo de genes necesarios para la producción de bioluminiscencia por *Vibrio fischeri* consiste de ocho genes denominados *luxA-E*, *luxG*, *luxI* y *luxR* (Engebrecht et. al., 1983). El gen *luxI* codifica para la autoinductor sintetasa; los genes *luxA* y *luxB* codifican para la luciferasa; *luxG* codifica para una flavín reductasa, en tanto *lux*C, *luxD* y *luxE*, codifican para un complejo multienzimático responsable de la síntesis de formaldehído, el cual es utilizado por la luciferasa. "Corriente arriba" de este operón y transcrito divergentemente, se encuentra el gen *luxR* que codifica para el regulador transcripcional LuxR responsable de la activación de la expresión de los demás genes *lux* (Whitehead, 2001) **(Figura 3)**.

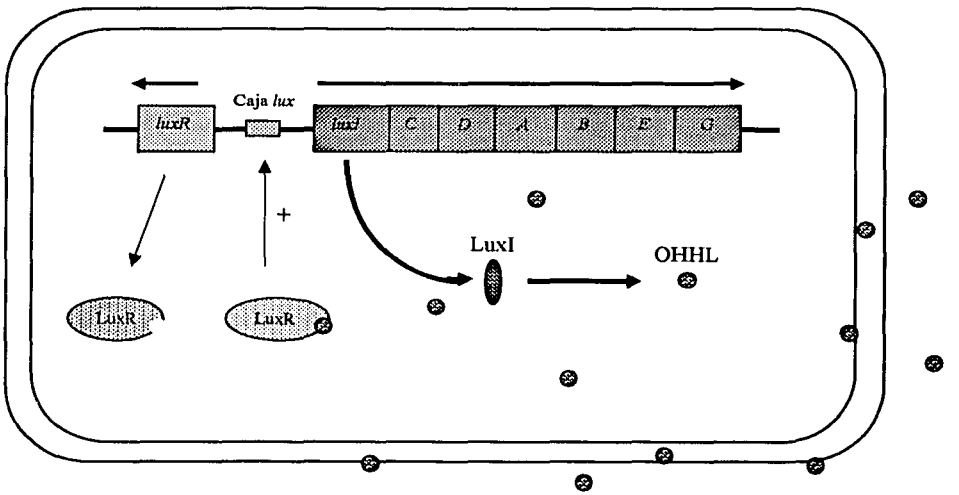

Figura 3. Regulación de la bioluminiscencia en *V. fischeri*. A alta densidad celular, se alcanza una concentración crítica de OHHL, el cual se une a LuxR y estimula la transcripción del operón *luxICDABEG*.

La inducción de bioluminiscencia por *V. fischeri* se dispara por la acumulación en el órgano de luz de un calamar o en un matraz, del autoinductor *N*-3-oxohexanoil-L-homoserin lactona (OHHL), sintetizado por LuxI. Cuando la densidad celular es alta, el autoinductor alcanza una concentración umbral e interactúa con LuxR, provocando un cambio conformacional en la proteína que le permite unirse a una secuencia específica de 20 pares de bases denominada caja *lux*, con la secuencia 5'-ACCTGTAGGATCGTACAGGT-3', centrada a –40pb del inicio transcripcional de *luxI*, siendo el complejo capaz de activar la transcripción del operón *luxICDABEG* (Devine et. al., 1989; Egland y Greenberg, 1999). Este mecanismo de autoinducción permite la acumulación intracelular del ARN que codifica tanto las funciones de bioluminiscencia como de síntesis de OHHL llevando a una producción continua de luz cuando se dispara el proceso (**Figura 3**). En la presencia de niveles bajos de OHHL, LuxR activa la transcripción de *luxR*, sin embargo cuando la concentración de autoinductor es alta, LuxR reprime la transcripción de *luxR*, lo cual provee de una autolimitación al sistema, ya que se requiere de una considerable cantidad de energía para la producción de bioluminiscencia que no es conveniente gastar si no se encuentran condiciones adecuadas (Shadel y Baldwin, 1991).

Familia LuxR de reguladores transcripcionales

La proteína LuxR de *Vibrio fischeri* posee 250 aminoácidos y requiere de las chaperonas GroESL para plegarse en una forma activa (Kaplan y Greenberg, 1987; Adar y Ulitzur 1993). Se ha demostrado que LuxR se localiza en la cara citoplásmica de la membrana interna y está compuesta de dos dominios: 1) un amino terminal que contiene una región de unión a N-3-oxohexanoil-homoserin lactona (OHHL), lo cual se encontró clonando solo la parte 5' del gen y observando que células que expresaban la proteína truncada retenían autoinductor marcado radioactivamente y; 2) un carboxilo terminal con una región de unión a ADN y de activación transcripcional, lo cual se encontró purificando esta parte de la proteína y observando su interacción directa con ADN así como la activación independientemente de la presencia de autoinductor (Kolibachuk y Greenberg, 1993; Slock et. al., 1990; Hanzelka y Greenberg, 1995) (**Figura 4**).

En general, los promotores que responden a reguladores transcripcionales de la familia LuxR, contienen una secuencia repetida inversamente de 18 a 20 pares de bases, centrada aproximadamente a -40pb del inicio de transcripción, a la cual se le ha llamado caja *lux* o caja *las* en *P. aeruginosa* (Stevens et. al., 1994; Pesci et. al., 1997). Esta secuencia posee el consenso CT-N_{12}-AG, pero se desconoce cuales son las bases nucleotídicas importantes para la especificidad de unión de las diferentes proteínas de la familia LuxR (Whiteley et. al., 1999).

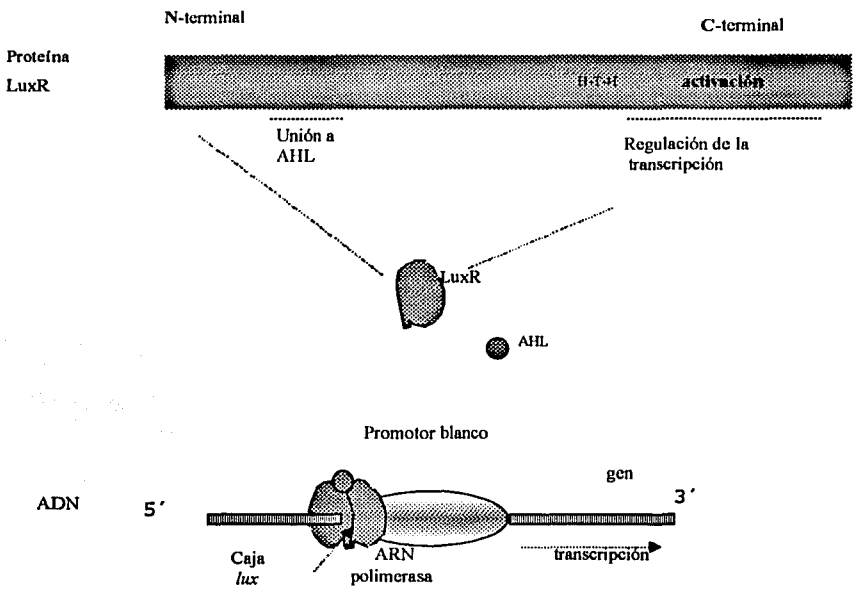

Figura 4. Dominios de la proteína LuxR y modelo de activación de la transcripción por dicha proteína.

El alineamiento de LuxR con sus homólogos conocidos revela una identidad 18 a 25% y solo cinco residuos son completamente conservados en todos los miembros de la familia de los cuales está disponible la secuencia (Whitehead et. al., 2001). Con tan baja identidad, no resulta sorprendente que en años recientes se halla determinado que algunas proteínas de la familia

LuxR poseen diferentes mecanismos de regulación. Por ejemplo los reguladores TraR de *Agrobacterium tumefaciens* y CarR de *Erwinia carotovora* se unen a su secuencia blanco solo en presencia de su autoinductor, el cual promueve su multimerización y afinidad (Zhu y Winans, 1999; Luo y Farrand, 1999; Welch et. al., 2000). Por otro lado, el regulador ExpR de *Erwinia chrysanthemi* se une a su propio promotor en ausencia de su autoinductor reprimiendo su transcripción y se disocia en la presencia de éste para permitir su expresión. ExpR puede También ser un regulador positivo de otros promotores a los que se pega tanto en ausencia como en presencia de autoinductor (Nasser et. al., 1998; Reverchon et. al., 1998).

Síntesis de *N*-acil homoserin lactonas.

Los autoinductores mas comunes en bacterias gram negativas, poseen una estructura general que consta de un grupo acilo de longitud variable unido por un enlace amídico a un grupo homoserina lactonizado (Fuqua y Greenberg, 1998). Estudios genéticos de *luxI* y otros genes relacionados han indicado que codifican para las enzimas responsables de la síntesis de los autoinductores, sin embargo fue hasta años recientes que se comprobó que las proteínas TraI conjugada con seis residuos de histidina y LuxI conjugada con la proteína de unión a maltosa, son capaces de sintetizar sus autoinductores correspondientes *in vitro* (Moré et. al., 1996; Schaefer et. al., 1996).

Los substratos requeridos para la biosíntesis de acil homoserin lactonas son S-adenosil metionina (SAM) y la proteína acarreadora de grupos acilo (ACP) conjugada con un ácido graso (Eberhard et. al., 1991; Moré et. al., 1996; Schaefer et. al., 1996). El modelo descrito para la biosíntesis propone que SAM se une al sitio activo de LuxI y que el grupo acilo se transfiere a dicho complejo desde un acilo-ACP. El grupo acilo entonces forma un enlace amídico con el grupo amino de SAM. La lactonización resulta en la síntesis del acil-HSL y 5′-metiltioadenosina (**Figura 5**).

Los autoinductores sintetizados por proteínas de la familia LuxI conocidos a la fecha, varían en el tamaño y la composición de sus grupos acilo. Las cadenas pueden ser desde cuatro a 14 átomos de carbono de largo, pueden contener dobles enlaces y a menudo grupos oxo o

hidroxilo en el carbono 3. Algunos homólogos de LuxI pueden producir mas de un autoinductor al tomar ácidos grasos de diferente longitud, y aunque el papel fisiológico de los que se producen minoritariamente en cada especie no ha sido determinado, se ha propuesto que pueden ser antagonistas para otros sistemas detectores de quórum o moduladores de la respuesta (Zhu et. al., 1998).

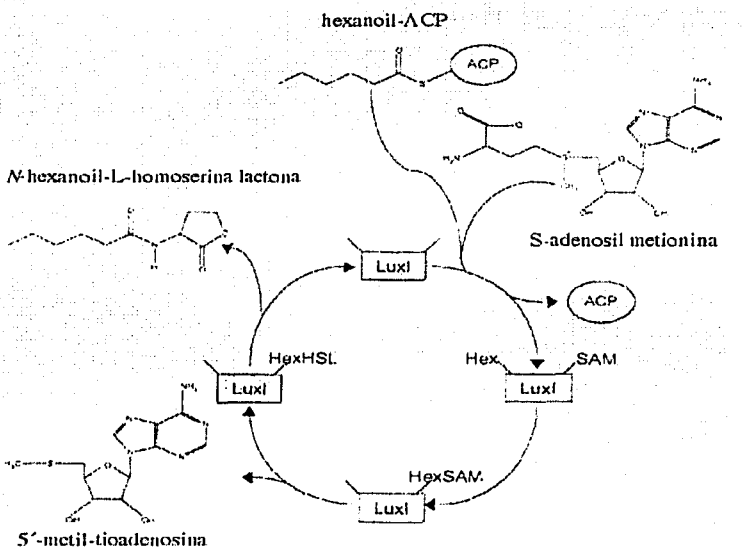

Figura 5. Modelo de la biosíntesis de HHL catalizado por LuxI en *V. fischeri*, el cual puede ser aplicado a otros sistemas tipo LuxI.

Sistemas detectores de quórum en *Pseudomonas aeruginosa*.

En *P. aeruginosa* existen al menos dos sistemas tipo detectores de quórum: *las* y *rhl*, los cuales regulan la expresión de factores de virulencia (Pesci et. al., 1997). El sistema *las* esta formado por el regulador LasR y el autoinductor *N*-3-Oxo-dodecanoil-homoserin-lactona (OdDHL) sintetizado por la proteína codificada por *lasI*. El complejo regula la transcripción de

las elastasas A y B, proteasa alcalina, exotoxina A, el desarrollo de biopelículas, el gen *rhlR* y el sistema de secreción *xcp* (Passador et. al., 1993; Toder et. al., 1991; Gambello et. al., 1993; Chapon-Hervé et. al., 1997; Davies et. al., 1998). El sistema *rhl,* formado por el regulador RhlR y el autoinductor *N*-butanoil-homoserin-lactona (BHL), el cual es sintetizado por el producto de *rhlI*, regula la producción de monoramnolípido, el cual es sintetizado por la ramnosil transferasa 1 codificada por los genes *rhlAB,* la producción de diramnolípido, sintetizado por la ramnosil transferasa 2 codificada por el gen *rhlC*, la producción de piocianina, cianuro de hidrógeno, lectinas, proteasa alcalina, quitinasas y la expresión de los genes *lasB* y *rpoS* (Rahim et. al., 2001; Ochsner et. al., 1994b; Pearson et. al., 1997; Brint y Ohman, 1995). Los genes *rhlABRI* se localizan en un mismo locus formando un regulón en el cual *rhlA* y *rhlB* se transcriben juntos. Corriente abajo de estos genes y en la misma dirección se encuentran los genes *rhlR* y *rhlI* (Ochsner et. al., 1994a; 1994b) (**Figura 6**).

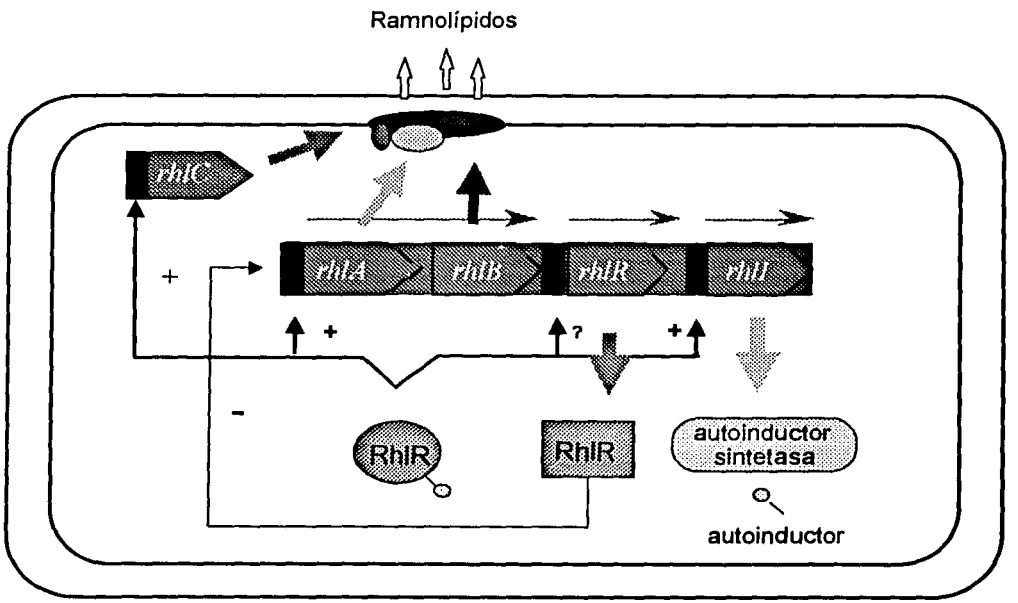

Figura 6. Modelo regulatorio de la producción de ramnolípidos en *P. aeruginosa.*

Debido a que los dos sistemas detectores de quórum regulan algunos factores de virulencia en común, se iniciaron estudios para determinar como interactúan entre sí. El sistema *rhl* es funcionalmente dependiente del sistema *las*, ya que la expresión de *rhlR* es activada por LasR-OdDHL, de tal manera que la activación del sistema *las*, causa la subsecuente activación del sistema *rhl* y juntos regulan la transcripción de los genes de sus respectivos regulones (Pesci et. al., 1997; Latifi et. al., 1996) (**Figura 7**).

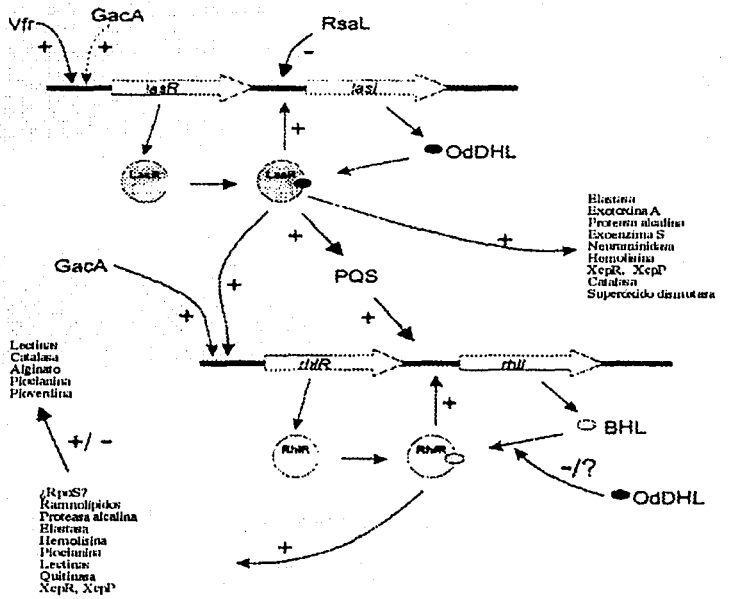

Figura 7. Modelo de regulación en *P. aeruginosa* mostrando los dos sistemas detectores de quórum, algunos factores de virulencia afectados y otros reguladores importantes.

Los genes que responden al sistema detector de quórum en *P. aeruginosa*, han sido clasificados dependiendo de a cual autoinductor responden y a si la expresión es temprana o tardía (Whiteley et. al., 1999). Los experimentos fueron hechos en una mutante *lasI/rhlI* y se

desconocen los factores por los cuales algunos genes solo se expresan en fase estacionaria incluso en presencia de autoinductores. Diversos compuestos regulados por el sistema detector de quórum solo son producidos bajo condiciones de limitación de nutrientes (Whiteley et. al., 1999), sin embargo, los mecanismos genéticos responsables de la respuesta a las condiciones de cultivo en relación con el sistema detector de quórum no han sido determinados.

Relación de los sistemas *las* y *rhl* con otros sistemas reguladores

Recientemente se ha encontrado que diferentes sistemas de regulación genética controlan la virulencia de *P. aeruginosa* sumándose a la regulación por *las* y *rhl* y en relación con éstos. Primeramente, se ha demostrado, que la expresión de *lasR* es dependiente de VFR (virulence factor regulator por sus siglas en inglés), un homólogo de la proteína receptora de adenosín monofosfato cíclico (CRP) de *E. coli*, la cual activa una gran cantidad de promotores incluyendo los necesarios para la utilización de fuentes alternativas de carbono (Ebright R. H., 1993). VFR se une a una secuencia consenso tipo CRP localizada corriente arriba de *lasR* (Albus et. al., 1997). Por otro lado, el activador global GacA de la familia de dos componentes, activa la expresión de *rhlR*, modula la expresión de BHL y los fenotipos controlados por el sistema *rhl*. GacA tambien afecta los niveles de expresión de *lasR* aunque no los de OdDHL (Reimmann et. al., 1997) (**Figura** 7).

La interrupción del gen *ppK*, un gen involucrado en la síntesis de polifosfato inorgánico, tiene un efecto dramático en la síntesis de BHL y OdDHL y como consecuencia en la expresión de factores de virulencia (Rashid et. al., 2000). Se ha reportado que el complejo RhlR-BHL es capaz de activar la transcripción de *rpoS*, el cual codifica para el factor sigma alternativo de fase estacionaria σ^s (Latifi et. al., 1996), sin embargo la regulación de *rpoS* por la respuesta detectora de quórum ha sido recientemente cuestionada, por lo que hacen falta mas estudios al respecto (Whiteley et. al., 2000). Por otro lado se demostró que la proteína RpoS funcional es importante para la producción de factores de virulencia que se conoce son regulados por los regulones *las* y *rhl*, como lectinas, catalasa, exotoxina A, piocianina y pioverdina (Suh et. al., 1999).

El principal efector de la respuesta astringente es el tetrafosfato de guanosina (ppGpp),

sintetizado por RelA que en *E. coli* provoca un cambio global en el metabolismo incluyendo un aumento en la expresión de *rpoS*. Cuando se exploró la posibilidad de que RelA tuviera un efecto sobre la respuesta detectora de quórum, se encontró que al sobre-expresar *relA* además de incrementarse los niveles de RpoS, ocurre una expresión prematura tanto de *lasR* como de *rhlR* así como la acumulación prematura de OdDHL y BHL, lo que sugirió que la respuesta astringente puede activar los dos sistemas detectores de quórum independientemente de la densidad celular (van Delden et. al., 2001).

Otros factores que modulan la respuesta detectora de quórum son los siguientes: 1) El producto del gen *rsaL* localizado corriente abajo de *lasR* y transcrito en sentido contrario a éste, reprime la transcripción de *lasI* a baja densidad celular (de Kievit et. al., 1999); 2) El gen *qscR* de *Pseudomonas aeruginosa*, codifica para una proteína homóloga de LasR y RhlR, la cual reprime la expresión de *lasI*, sugiriendo que los genes controlados por la respuesta detectora de quórum no son activados en condiciones donde no son necesarios y la proteína QscR está presente (Chugani et. al. 2001) y; 3) La molécula 2-heptil-3-hidroxi-4-quinolona (PQS), induce la expresión de *rhlI* y de *lasB* cooperativamente con BHL aunque aún no se conoce la manera en que pudiera estar regulando la respuesta detectora de quórum y si actúa en conjunción con LasR, RhlR o con otro regulador que no ha sido identificado (McKnight et. al., 2000).

IV. Antecedentes

Los genes *rhlAB* fueron aislados y caracterizados por Ochsner et. al. (1994a) quienes encontraron que se transcriben como un operón. Los mismos autores midieron la expresión de *rhlA* utilizando fusiones con *lacZ* en *P. aeruginosa*, encontrando que se transcribe principalmente en fase estacionaria y que depende de RhlR ya que en una mutante *rhlR* disminuyó drásticamente su expresión. Pearson et. al. (1997) mapearon el inicio transcripcional del operón *rhlAB en P. aeruginosa* y observaron que células de *E. coli* expresando *rhlR* retienen BHL marcado radiactivamente, por lo que proponen que existe una interacción directa entre RhlR y BHL. Dichos autores también demostraron que RhlR y BHL son suficientes para la expresión de una fusión *rhlA::lacZ* en *E. coli* y sugirieron que *rhlA* posee un promotor tipo σ^{54}. Rahim et. al. (2001) propusieron que RhlA es una proteína asociada a la membrana interna en tanto RhlB es la subunidad catalítica y contiene dos dominios transmembranales lo que sugiere que secreta los ramnolípidos al periplasma.

El gen *rhlR* fue aislado y caracterizado por separado por Ochsner et. al. (1994b) y por Brint y Ohman (1995). Ambos grupos propusieron que es un activador transcripcional de la familia LuxR, y que es necesario para la producción de ramnolípidos así como de otros factores de virulencia.

Latifi et. al. (1996) demostraron que la expresión de *rhlR* depende de *lasR* utilizando fusiones *rhlR::lacZ* en una mutante *lasR*, en tanto Pesci et. al. (1997) además de llegar a similares conclusiones utilizando fusiones *rhlR::lacZ* en ausencia o en presencia de *lasR* en el fondo genético de *E. coli*, encontraron que el autoinductor OdDHL bloquea la unión de BHL a RhlR lo cual inhibe la expresión de *rhlA*.

Debido al interés en el laboratorio de la Dra. Gloria Soberón Chávez de conocer la regulación de la síntesis de ramnolípidos, y dado que en otros trabajos no se realizó una caracterización detallada de los promotores de los genes *rhlA* y *rhlR*, ni se exploró el posible mecanismo de regulación por RhlR, en el presente trabajo se plantearon los siguientes objetivos.

V. Objetivos

Objetivo general

-Estudiar la regulación transcripcional de los genes *rhlA* y *rhlR* de *Pseudomonas aeruginosa* así como la interacción de la proteína RhlR con la región promotora de *rhlA*.

Objetivos específicos

-Determinar cuales son los factores involucrados en la expresión del gen *rhlA* y las condiciones necesarias para su expresión.

-Establecer él o los sitios de unión de la proteína RhlR a la región promotora del gen *rhlA* y las condiciones en que se presenta dicha interacción.

-Determinar cuales son los factores involucrados en la expresión del gen *rhlR*, sus inicios transcripcionales, y las condiciones necesarias para su expresión.

VI. Resultados y discusión.

Resultados I. Artículo aceptado en la revista Journal of Bacteriology.

Title: The *Pseudomonas aeruginosa rhlAB* operon is not expressed on the logarithmic phase of growth even in the presence of its activator RhlR and the autoinducer *N*-butanoyl-homoserine lactone.

Authors: Gerardo Medina, Katy Juárez and Gloria Soberón-Chávez*.

Address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.

*Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, UNAM. Apdo. Postal 510-3, Cuernavaca Morelos 62251, México. Phone : (52) (777) 3291634. Fax: (52) (777) 3172388. E-mail: gloria@ibt.unam.mx

Abstract

The *Pseudomonas aeruginosa rhlAB* operon encodes for the enzyme rhamnosyltransferase 1, which produces the biosurfactant mono-rhamnolipid; *rhlAB* induction is dependent on the quorum-sensing transcription activator RhlR complexed with the autoinducer N-butyryl-homoserine lactone (C4-HSL). In this work we studied *rhlAB* induction in *P. aeruginosa* and *Escherichia coli* backgrounds. We found that in both bacteria, its expression is not induced during the logarithmic phase of growth even on the presence of RhlR and C4-HSL. Additionally we found that *rhlAB* expression is partially σ^s-dependent.

Pseudomonas aeruginosa is an opportunistic pathogen causing serious nosocomial infections (5). The production of several of the virulence-associated traits produced by this bacterium is regulated at the level of transcription by the so-called quorum-sensing response (8, 30).

Rhamnolipids are biosurfactants produced by *P. aeruginosa* (13) that are regulated by the quorum-sensing response dependent on the transcription activator RhlR (16) and the autoinducer (AI) *N*-butyryl homoserine lactone (C4-HSL) synthesized by RhlI (17). The *rhlAB* operon encodes rhamnosyltransferase 1 that is responsible for mono-rhamnolipid production from TDP-L-rhamnose and β-hydroxy-fatty acids (15), and *rhlC* encodes rhamnosyltransferase 2 that produces di-rhamnolipid using mono-rhamnolipid and TDP-L-rhamnose as substrates (22). The model for the transcription activation of *rhlAB* and *rhlC* is that with increasing bacterial cell-density the concentration of C4-HSL reaches a threshold level and then attaches to the transcription activator RhlR (16). The RhlR/C4-HSL complex activates the transcription of the *rhlAB* operon (18, 20) and *rhlC* (22).

P. aeruginosa contains a second quorum sensing regulon, consisting of the transcription regulator LasR, which is activated by the AI *N*-3-oxododecanoyl homoserine lactone (3-o-C12-HSL). The transcription of several genes encoding virulence-associated traits is activated by the Las system (8, 31), and it has a central role in the transcription of *rhlR* (12, 20).

The aim of this work was to determine whether the expression of the *rhlAB* operon is only dependent on the presence of RhlR and C4-HSL or other regulatory elements participate. To do this we studied *rhlAB* expression along the growth curve of *P. aeruginosa*, PAO1 with plasmid pECP61.5 (*rhlA::lacZ, ptac-rhlR,* Table 1) adding C4-HSL and 0.1 mM IPTG from the onset of the culture on the phosphate limited PPGAS medium used to produce rhamnolipids (32). It was apparent that *rhlAB* promoter is not expressed during the logarithmic phase of growth even in the presence of C4-HSL and RhlR, detected by Western immunobloting (Fig. 1). To ensure that RhlR expression was not a limiting factor IPTG was added from the beginning of the culture. We detected the presence of RhlR protein as early as the second hour after induction, corresponding to

a turbidity of 0.1 at 600 nm, while the expression of the *rhlA::lacZ* fusion was apparent until six hours after induction, when the culture has an optical density of 1.7 (Fig. 1).

We determined that the lack of *rhlA::lacZ* expression during the exponential phase of growth was not due to a limitation of C4-HSL concentration, since the same results were obtained when a 10 fold concentration of this autoinducer was used (data not shown).

To carry out the immunodetection of RhlR, New Zealand rabbits were immunized with a protein fusion Thio-RhlR in order to produce polyclonal antibodies. To construct a Thio-RhlR protein fusion, the *rhlR* gene was PCR amplified. The product was digested with *Kpn*I and *Sal*I and cloned into plasmid pThioC (Invitrogen) digested with the same enzymes. To detect the protein *P. aeruginosa* or *E. coli* cultures, as stated, were grown until they reached the indicated turbidity at 600 nm (Figs. 1 and 2). At this point the cells were collected and lysed by boiling for 5 min in loading buffer (23). Equal amounts of protein from each lysate were separated in a 14% (w/v) SDS/PAGE. The proteins were transferred by electroblotting from the gel to Hybond-C nitrocellulose membranes (Amersham Life Science Corp.). RhlR was detected by using the rabbit polyclonal antiserum raised against ThioR-RhlR.

Different *P. aeruginosa* genes regulated by the quorum-sensing response have been classified depending on their response to 3-o-C12-HSL and C4-HSL and the time course of their induction (28). Several of the genes identified were not expressed during the exponential phase of growth (classified as type 2 and 4). According to this study (28) the *rhlAB* promoter was classified as a type 3 promoter. This type of promoters responds only in the presence of both autoinducers (AIs) and are expressed from the onset of the culture (28). All genes regulated by RhlR and C4-HSL are expected to be dependent on both AIs in the experimental conditions of *Whiteley et al* (28), since the expression of *rhlR* is dependent on 3-o-C12-HSL (12, 20). The results presented here are in contradiction with this observation since we found that *rhlAB* is not expressed in the logarithmic phase of growth even in the presence of RhlR and C4-HSL. To determine whether the differences in our results with those previously reported (28) could be due to the lack of expression of a factor, different from RhlR, depending on the presence of 3-o-C12-HSL, we studied *rhlAB* expression on PPGAS medium

supplemented with both autoinducers. Our data show that in the PAO1 background *rhlAB* is not expressed during the logarithmic phase of growth even when C4-HSL and 3-o-C12-HSL are present (Fig. 1).

Another possibility to explain the discrepancy between our results and the previously reported data (28) was the use of different culture media to study *rhlAB* expression. To explore this possibility we did the experiments on LB medium (14) as Whiteley *et al.* (28). We found that *rhlAB* was not expressed on the logarithmic phase of growth of PAO1 cells grown on LB medium even when it was supplemented with C4-HSL or both C4-HSL and 3-o-C12-HSL (Fig. 1). It is apparent that *rhlAB* is expressed at a lower level on LB medium, since we obtained considerably lower β-galactosidase activities than those obtained when this bacterium is grown on PPGAS medium (Fig. 1). It can be concluded from our results that *rhlAB* operon belongs to the type 4 group of quorum-sensing regulated genes according to the classification of Whiteley *et al.* (28). It is possible that *rhlAB* expression during the exponential phase of growth reported (28) was due to a particular status of the quorum-sensing response in the *lasI, rhlI* derived PAO1 mutant, supplemented with both AIs, that was used in that study.

Several gene products that exert a negative effect on *P. aeruginosa* quorum-sensing response have been reported (2, 4, 7, 21, 25, 29). The products of these genes have recently been postulated to participate in preventing the early expression of type 2 and 4 quorum-sensing regulated genes (19). Most of these negative regulators, as RpoS (25, 29), RsmA (21), DksA (2), and QscR (4), exert their negative regulatory effect through the repression of one or both genes encoding autoinducer synthetases *lasI* or *rhlI*, and are thus not expected to have any repressing activity when AIs are supplemented to the culture medium, as in the case we are studying. It has very recently been shown that *P. aeruginosa* MvaT has a negative effect on the expression of different quorum-sensing regulated traits and could have a minor role in preventing the expression of the *lecA* gene during the exponential phase of growth in the presence of AIs (7, 19). The mechanism of MvaT control of quorum-sensing regulated genes expression is unknown, but it is known that this regulator is only present in Pseudomonads (7).

To determine whether the regulatory elements (a repressor or the lack of an activator) that prevent *rhlAB* expression during logarithmic phase of growth were only present in the *P. aeruginosa* genetic background we studied the kinetics of *rhlAB* expression along the growth curve of *Escherichia coli* DH5α with plasmid pECP61.5. It was apparent that this operon was not expressed during the exponential phase of growth, as was found in *P. aeruginosa,* even in the presence of C4-HSL and RhlR (Fig. 2). These results show that the presence of RhlR and C4-HSL is a necessary condition for *rhlAB* expression, but that there is a regulatory element that prevents the expression of this operon during the exponential phase of growth.

We further characterize the exponential silencing of the *rhlAB* promoter in an *E. coli rpoS* mutant, and we found that it also occurs, but that the level of *rhlA::lacZ* expression on the stationary phase of growth was considerably lower (Fig. 3A), suggesting that in *E. coli* this sigma factor is involved, directly or indirectly, in *rhlAB* expression during the stationary phase of growth.

It has been reported that in *P. aeruginosa* a *rpoS* mutation causes an increased pyocyanin production (25, 29), presumably due to an increased production of C4-HSL by this mutant (29). However, some quorum-sensing regulated traits, such as exotoxin A, are produced at a lower level (25), and the production of lectins, for example, are completely abolished, since the *lecA* gene has a σ^s-dependent promoter (30).

We constructed a PAO1 *rpoS::Gm* mutant, called PAS1 (Table 1) by the following procedure: A PCR product containing the *rpoS* gene was digested with *Pst*I and *Sma*I and subsequently ligated into pBluescript-KS-II (Stratagene). A 1.1-kb Gm cassette from pBSL141 (1) was inserted into the unique *Hinc*II site of pCOC1 within *rpoS*. The resulting knockout construct was transformed into *P. aeruginosa* and mutants were selected as gentamicin resistant and carbenicillin sensitive. PCR and Southern blot analysis were performed to confirm the presence of the Gm cassette within the chromosome of *P. aeruginosa rpoS* mutant (data not shown).

Strain PAS1 produces, as expected, high levels of pyocyanin measured as described previously (6) (PAO1 produces 0.46 μg/ml, while PAS1 produces 0.71 μg/ml at 8 hours of growth in PPGAS medium at 37°C). PAS1 mutant produces similar rhamnolipids level as PAO1, determined by the orcinol method (3) (the wild type strain produces 172

μg/ml, while the *rpoS* mutant produces 161 μg/ml at same conditions used to measure pyocyanin). We found that in PAS1, as in *E. coli rpoS* mutant, the *rhlAB* operon is not expressed in the exponential phase of growth and that its expression is significantly lower in stationary phase (Fig. 3B). These results show that *rhlAB* expression is partially σ^s-dependent, even in the presence of increased C4-HSL concentration (31).

It was reported that the expression of the *E. coli relA* gene, encoding the ppGpp synthase, during the exponential growth of *P. aeruginosa* PAO1 causes an early expression of different traits involved in the quorum sensing response including AIs (26). One possible explanation for the exponential silencing of the *rhlAB* promoter is that it is dependent on the stringent response and hence this operon will not be expressed when there is not limitation of bacterial growth. This would explain the dependence of its expression on σ^s, since it has been shown in *E. coli* that gene expression by this alternative sigma factor is tightly linked to the stringent response (9, 11). We have preliminary evidence suggesting that, both in *P. aeruginosa* as in *E. coli*, the *rhlAB* promoter is not silenced during the exponential growth in minimal media (data not shown). These results suggest that *P. aeruginosa* nutritional status, maybe sensed through the stringent response, is involved in the regulation of *rhlAB* expression.

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TABLE 1. Strains and plasmids used in this study

Strain	Relevant characteristics	Reference
Pseudomonas aeruginosa		
PAO1	wild type strain	10
PAS1	PAO1 *rpoS::Gm* mutant	This study
Escherichia coli:		
DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi1 relA1*	23
MC4100	F- Δ(*argF-lac*) *thiA*	24
JV1065	MC4100 derivative *λpir rpoS13::Tn10*	27
Plasmids		
pECP61.5	contains an *rhlA::lacZ* translation fusion and *rhlR* under *ptac*, Ap^r	18

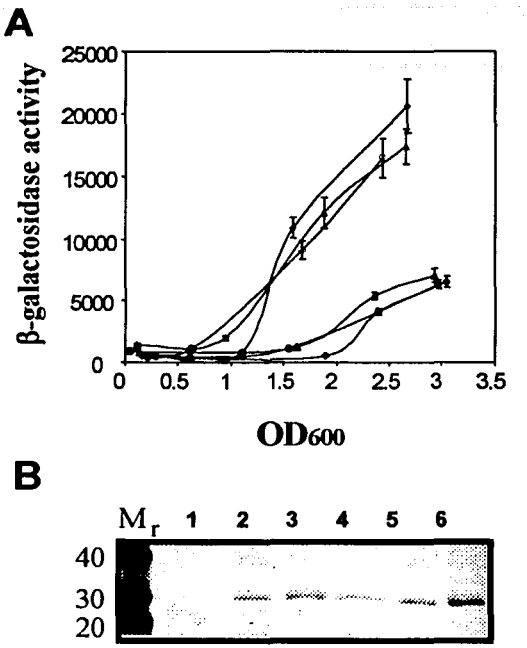

Figure 1. A) Expression of the *rhlA::lacZ* translational fusion encoded in plasmid pECP61.5 in *Pseudomonas aeruginosa* PAO1 strain (rhombus), as a function of the optical density at 600 nm of a culture grown on PPGAS (open symbols) and LB (closed symbols) media at 37°C, and on these media supplemented with 10 μM C4-HSL and 0.1 mM IPTG (circles), or 10μM of both C4-HSL and 3-o-C12-HSL and 0.1 mM IPTG (triangles). β-galactosidase activity is expressed in Miller units (14). B) Immunoblotting of the RhlR protein along the growth curve of strain PAO1 on PPGAS medium (32) shown in A). Lanes 1), 2) and 3) show RhlR expressed in cells grown on this medium at an optical density at 600 nm of approximately 0.1, 0.7 and 2.4 respectively. Lanes 4), 5) and 6) present RhlR expressed in cells grown on PPGAS supplemented with 10 μM C4-HSL and 0.1 mM IPTG at the same cell densities. The size of the molecular markers is shown at the left side of the lane marked as Mr.

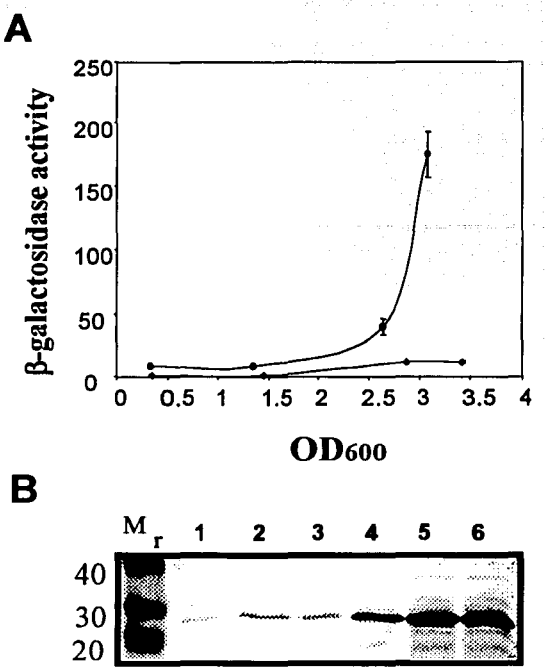

Figure 2. (A) Expression of the *rhlA::lacZ* translational fusion encoded in plasmid pECP61.5 in *E. coli* strain DH5α as a function of the optical density at 600 nm of a culture grown at 37°C on LB medium (14) (rhombus); and on this medium supplemented with 10 μM C4-HSL and 0.1 mM IPTG (circles). β-galactosidase activity is expressed in Miller units (14). (B) Immunoblotting of the RhlR protein along the growth curve of strain DH5α shown in A). Lanes 1), 2) and 3) show RhlR expressed in cells grown on LB medium) at an optical density at 600 nm of approximately 0.4, 1.6 and 3.4, respectively. Lanes 4), 5) and 6) present RhlR expressed in cells grown on LB supplemented with 10 μM C4-HSL and 0.1 mM IPTG at the same optical densities. The size of the molecular markers is shown at the left side of the lane marked as Mr.

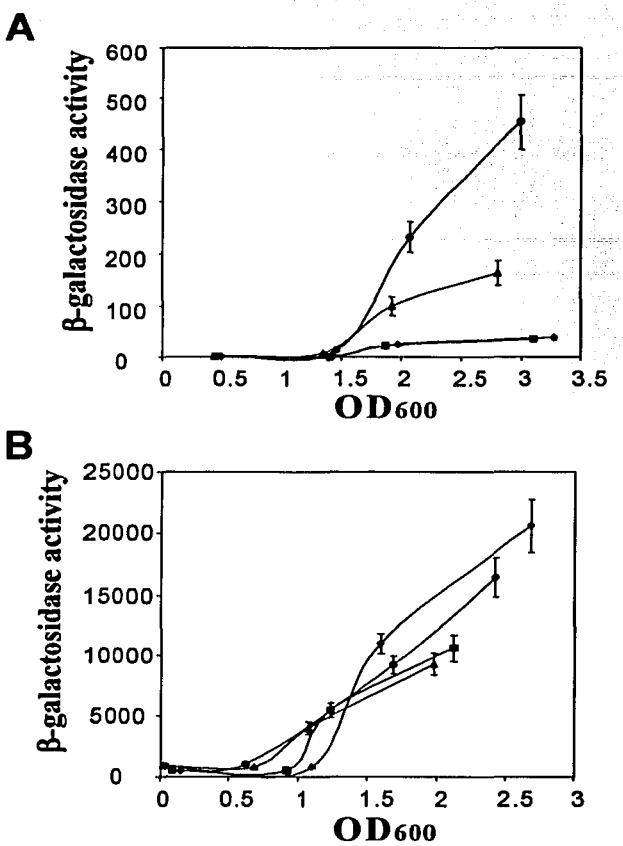

Figure 3. Effect of an *rpoS* mutation on the expression of the *rhlA::lacZ* fusion encoded in plasmids pECP61.5 in *E. coli* (A) and *P. aeruginosa* (B) as a function of the optical density at 600 nm of cultures grown on LB (14) and PPGAS (32) media, respectively. Symbols correspond to: *E.coli* strain MC4100 grown on LB (rhombus) and on this medium supplemented with 10 μM C4-HSL and 0.1 mM IPTG (circles); its derived *rpoS* mutant JV1065 on LB (squares) and on the same medium supplemented with 10 μM C4-HSL and 0.1 mM IPTG (triangles). *P. aeruginosa* PAO1 on PPGAS (rhombus) and on the same medium supplemented with 10 μM C4-HSL and 0.1 mM IPTG (circles); its *rpoS* derived mutant PAS1 on PPGAS (squares) and on the same medium supplemented with 10 μM C4-HSL and 0.1 mM IPTG (triangles). β-galactosidase activity is expressed in Miller units (14).

Resultados II. Artículo sometido a revisión a la revista Journal of Bacteriology.

Title: Transcriptional regulation of *Pseudomonas aeruginosa rhlR* encoding a quorum sensing regulatory protein

Authors: Gerardo Medina, Katy Juárez, Rafael Díaz, and Gloria Soberón-Chávez*.

Address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Apdo. Postal 510-3, Cuernavaca, Mor. 62251, México.

*Corresponding author mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, UNAM. Apdo Postal 510-3, Cuernavaca Morelos 62251, México. Phone : (52) (73) 291634. Fax: (52) (73) 172388. E-mail: gloria@ibt.unam.mx

ABSTRACT

The *Pseudomonas aeruginosa rhlR* gene encodes the transcription regulator RhlR that has a central role in the quorum sensing response. Different compounds involved in bacterial pathogenesis are regulated at the transcription level by the two quorum-sensing response systems, Las and Rhl. The expression of *rhlR* has been reported to be under the control of the Las system, but its transcription regulation has not been studied in detail. Here we characterize the *rhlR* promoter region, showing that it presents four different transcription initiation sites, two of which are included in the upstream gene (*rhlB*) coding region. We found that *rhlR* expression is partially LasR-independent under certain conditions and that its expression from these multiple promoters depends on LasR and on different regulatory proteins such as Vfr and RhlR itself, and also on the alternative sigma factor σ^{54}.

INTRODUCTION

Pseudomonas aeruginosa can be isolated from many different habitats including water, soil and plants (4, 12), but it is also an opportunistic human pathogen causing serious nosocomial infections (4). The production of several virulence associated compounds (5), including rhamnolipids, is coordinately regulated by a mechanism called quorum-sensing, which depends on the production of two autoinducers: butyryl-homoserine lactone (C4-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-o-C12-HSL) that coupled with RhlR and LasR proteins respectively, activate gene expression. RhlI synthesize the former autoinducer and LasI the latter (7, 8, 14). The quorum-sensing response that depends on LasR (coupled with 3-0-C12-HSL) promotes the transcription activation of several virulence associated traits (2, 6, 10, 15, 22, 23, 29) including the gene coding for the transcription regulator RhlR (15, 23). The second quorum-sensing genetic circuit responds to RhlR (20) coupled with C4-HSL (21) and promotes the expression, among others (3), of the *rhlAB* operon encoding rhamnosyltransferase 1 enzyme (19) and the *rhlC* gene encoding rhamnosyltransferase 2 (24). These rhamnosyltransferases are the enzymes responsible for rhamnolipid biosynthesis (16).

The LasR and RhlR proteins belong to the LuxR family of transcription regulators that bind to specific DNA sequences, called lux boxes (7, 8). In the case of *P. aeruginosa* these sequences have been called las boxes (32).

The *rhlR* gene is encoded immediately downstream of the *rhlAB* operon, and has been reported to be activated by LasR (15, 22). A promoter sequence has been predicted on the *rhlB-rhlR* intergenic region (20). The role of LasR in *rhlR* transcription was concluded from two types of experimental evidence. First a *P. aeruginosa lasR* mutant does not express an *rhlR::lacZ* fusion when grown on LB medium and, second, *Escherichia coli* expresses an *rhlR::lacZ* fusion at a low, but significant level in the presence of LasR and 3-O-C12-HSL.

The aim of this work was the detailed characterization of the *rhlR* promoter region and the identification of the different elements that participate in the regulation of its expression. In this study we used a culture medium (PPGAS) where rhamnolipids production is high (35), so presumably *rhlR* expression is also high. On this condition we detected four *rhlR* transcription initiation sites, two within the *rhlB*-coding region. It

was also apparent that *rhlR* can be expressed at a significant level in the absence of LasR, and that, besides this quorum sensing regulator, different transcription activators, like Vfr and RhlR itself, as well as the alternative sigma factor σ^{54}, participate in its expression from these multiple promoters. Two putative las boxes were detected in the *rhlR* regulatory region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used are listed in Table 1. Bacterial strains were cultured in Luria Bertani medium (LB, 18) or in phosphate-limited peptone-glucose-ammonium salts medium pH 7.2 (PPGAS, 34) containing NH_4Cl_2 (0.02 M), KCl (0.02 M), Tris-HCl (0.12 M), $MgSO_4$ (0.0016 M), glucose (0.5%), and peptone (1%), as stated. Bacto-agar was added to 1.5% for solid media. When necessary isopropylthio-β-D-galactoside (IPTG, 0.1 mM) was added to the medium. Antibiotics (Sigma Chemical Co.) were used to supplement selection media at the following concentrations in μg/ml for *E. coli* and *P. aeruginosa*, respectively: ampicillin 150 and not used; carbenicillin not used and 250; tetracycline 15 and 100; and gentamicin not used and 200. β-galactosidase activity was determined as reported (18), one Miller unit corresponds to 1 nM of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute and per optical density at 600 nm. The presented data represent the triplicate measurements of two independent experiments.

Enzymes and reagents. Plasmids were purified using the Wizard plus minipreps DNA purification system (Promega corporation Madison WI). DNA bands were cut from agarose gels and purified with Geneclean III kit (Bio101 La Jolla CA). Enzymes were purchased from New England Biolabs (Beverly Mass.) or Roche Diagnostics Corporation (Indianapolis IND), and used according with the manufactures' instructions. DNA sequencing reactions were done using the Sequenase kit (Amersham Life Science Corp. Cleveland, Ohio). Oligonucleotides were radioactively labeled with the T4 polynucleotide kinase (Amersham Life Science Corp. Cleveland, Ohio), using [γ-^{32}P]ATP (Amersham International) as substrate. Blunt ended DNA fragments were obtained with the Klenow fragment of DNA polymerase I (Amersham Life Science Corp. Cleveland, Ohio). 3-oxo-dodecanoyl-homoserine lactone was purchased from Quorum Sciences Inc. (Coralville, IA)

Nucleic acid techniques. DNA manipulation was done as reported (26). Plasmid electroporation into *P. aeruginosa* was done as reported (27).

Primer extension analysis. The different *P. aeruginosa* strains analyzed containing plasmid pPCS1002 were grown to an optical density of 1.5 at 600 nm and their total RNA was extracted using a GlassMAX RNA Microisolation Reagent Assembly (Life technologies Inc. Gaithesburg, MD). Primer extension reactions were done using the *C. thermophilus* polymerase for reverse transcription of the two-step RT-PCR kit (Roche Diagnostics Indianapolis, IN) according to the manufacturer's instructions. Reactions

were carried out at 60^{0}C. These experiments were done using two different oligonucleotides. Oligo-1 is located at +25 bp with respect of the *rhlR* translational start site and has the following sequence: 5'-CATCTCGCTACGCAAACCGTCCCACC-3'. Oligo-2 is located at -146 bp from the *rhlR* translation start site and has the sequence: 5'-CGCGCATCCCCCTCCCTATGACAAC-3'.

Plasmids construction. Plasmid pRD58-1 containing 288 bp of the *rhlR* upstream region fused to a promoterless *lacZ* gene was constructed isolating a 580 bp DNA fragment which contains the 288 bp of the *rhlR* regulatory region and part of the coding region by PCR, using the following oligonucleotides: 5'GCGCAAGCTTGTGGCGCTTGCTCGAGGACC-3' and 5'-CGCTCCAGACCACCATTTCC-3'. The PCR product was digested with *Hind*III and *Bam*H1 and cloned into the vector pMP220 (28). Plasmid pRD58-2 which contains 498 bp of the *rhlR* regulatory region fused to the *lacZ* gene of the vector pMP220 was constructed by ligating into this plasmid a fragment of 741 bp which include the *rhlR* upstream region and part of the coding region, that was purified from a *Pst*I-*Bam*H1 digestion of plasmid pUO58 which contains the entire *rhlA, rhlB, rhlR* and *rhlI* gene cluster (19).

Plasmid pMT1 that contains the *lasR* gene under the *lac* promoter was constructed amplifying a PCR fragment of 860 bp containing this gene, using plasmid pSB1075 (34) as template. The oligonucleotides used were: 5'-CCGGAATTCTCGGACTGCCGTACAAC-3' and 5'-GAAGGGCGAATTCCCGATCGCCAG-3'. The PCR product was cloned into pMOSblue plasmid (Amersham Pharmacia Biotec UK) which was later digested with *Kpn*I and *Hind*III endonucleases and the insert was subcloned into plasmid pUCP20 (31).

RESULTS AND DISCUSSION

The *rhlR* gene has four transcription start sites. The production of rhamnolipids by *P. aeruginosa* is highly dependent on culture medium composition (16), and we have determined that there is a correlation between the level of rhamnolipids produced and the level of *rhlR* expression (unpublished results). We used a culture medium (PPGAS) where high levels of rhamnolipids are produced (35) to study *rhlR* transcription regulation. The transcription start sites of the *rhlR* gene were determined by primer extension analysis (Figs. 1 and 2). To do these experiments plasmid pPCS1002 (22, Table 1) carrying a *rhlR::lacZ* fusion was transferred to different *P. aeruginosa* strains to enhance *rhlR* promoter expression and increase the sensitivity of the assay.

P. aeruginosa PAO1 *rhlR* gene presents four transcription start sites (P1 to P3 on Fig. 1 and P4 on Fig. 2), two of which (P3 and P4) are within the upstream *rhlB* gene coding region (Fig. 3A). The analysis of the *rhlR* regulatory sequence showed that three of the

detected start sites present sequences at -10 and -35 from the transcription initiation site that seem to be recognized by the RNA polymerase containing the σ^{70} subunit (P1, P2 and P4, Fig. 3A). The predicted promoter based on sequence analysis of *rhlB-rhlR* intergenic region (20) corresponds to the promoter designated by us as P2 (Fig. 3A). We suggest, based on the results described below, that the transcription start site at -159 (P3) is recognized by RNA-polymerase containing a σ^{54} subunit (P3, Fig. 3A).

The expression from the P1 and P4 *rhlR* transcription start sites is LasR dependent. It has been reported that *rhlR* transcription on LB medium depends on LasR activation (15, 23). In accordance with the reported results, we found that *rhlR* was expressed at a very low level in the *lasR* mutant PAOR1 when cells are grown on LB (Fig. 4A). However, we found that *rhlR* was expressed at a significant level on the *lasR* PAOR1 mutant when grown on PPGAS medium (Fig. 4A). This result shows that, when *P. aeruginosa* grows on PPGAS medium, *rhlR* expression can be activated in a LasR-independent manner. The PAOR1 *lasR* mutant is completely unable to produce rhamnolipids when grown on PPGAS (data not shown). These data suggest the presence in this condition of a point of *rhlR* posttranscriptional regulation that depends on a functional LasR protein, since it is not likely that LasR plays a regulatory role in the expression of *rhlAB* (17, 22).

We found that P1 and P4 initiation sites are not detected when the *lasR* PAOR1 mutant was grown on PPGAS medium (Figs. 1A and 2A). The P1 promoter has a characteristic structure of the promoters activated by regulators of the LuxR family (33), presenting the LasR binding site centered at -40 nucleotide from the transcription start site (Fig. 3A). Primer extension analysis in mutant PAOR1 confirms that *rhlR* expression from this promoter is completely dependent on LasR. There is not a clear explanation for the LasR-dependent P4 promoter transcription, since the position of the putative las boxes relative to this initiation site is not consistent with its direct activation by LasR. It is possible that this promoter is activated by LasR in an indirect manner. The role of the putative las box 2 is discussed below.

Vfr activates the expression of the P4 *rhlR* promoter. It has been reported that Vfr, a *P. aeruginosa* Crp homolog that regulates the expression of different virulence associated traits (30), directly activates *lasR* expression (1). However, primer extension

experiments done with the PAO1 derived *vfr* mutant PAO9001, show that P1, the LasR-dependent transcription start site is fully expressed (data not shown), while the P4 start site is not (Fig. 2). Accordingly with these results we found that *rhlR* expression is reduced in the *vfr* mutant PAO9001 (Fig. 4A).

Upstream of the P3 promoter and overlapping the P4 transcription start site we detected a putative Vfr binding site (Fig. 3A and 3B). The relative position of this Vfr binding site with respect to the P4 promoter, which is the detected point of Vfr regulation of *rhlR* transcription, is uncommon for a promoter activated by a Crp-like regulator. The Crp-activated promoters present a binding site centered at –40 or –60 nucleotides from the transcription start site, and when this protein binds downstream from the initiation site it acts as a repressor. Thus, additional evidence, as Vfr binding and footprinting analysis, is needed before it can be concluded that Vfr directly activates the *rhlR* P4 promoter.

The expression from P3 *rhlR* transcription start site is σ^{54} dependent. It has been reported that a *P. aeruginosa rpoN* mutant is unable to produce rhamnolipids (19, 22). To study the influence of the σ^{54} factor on *rhlR* expression, we used the *P. aeruginosa* PAK derived *rpoN* mutant PAKN1 (13, Table 1). We found that mutant PAKN1, when grown on PPGAS, expressed *rhlR* at a significantly lower level than the wild type strain PAK (Fig. 4B), showing that this alternative sigma factor is involved in *rhlR* expression.

Comparing the *rhlR* transcription start sites present in PAK and PAKN1 strains grown on PPGAS, we found that the only promoter which is completely dependent on σ^{54} is P3 (Fig. 1). Upstream of the P3 transcription initiation site we found sequences with the nucleotides and positions required to be recognized by the RNA-polymerase containing a σ^{54} subunit (Fig. 3A).

All σ^{54}-dependent promoters are activated by proteins belonging to the NtrC family. The activator protein of the P3 promoter and its DNA binding site remain to be identified.

Expression from the *rhlR* P2 transcription start site is repressed by RhlR. We detected an increased level of *rhlR* expression in the *rhlR* mutant 65E12 (20, Table 1) compared to the PG201 wild type strain, when grown on LB medium (Fig. 4C). The P2 promoter is the only detected *rhlR* active promoter on all the studied strains and mutants when grown on LB (data not shown). These data suggest that RhlR plays a negative role on the expression of the P2 transcription start site of its own gene.

When mutant 65E12 was grown on PPGAS we detected an increased transcription starting from the P2 promoter during the stationary phase of growth (Fig. 1), but this increment was not reflected in an increased level of expression of the *rhl::lacZ* fusion (Fig. 4C). These data suggest that RhlR also regulates its own gene expression when cells are grown on PPGAS by reducing the level of transcription from the P2 promoter. It seems though, that the contribution of P2 promoter to the expression of *rhlR*, when cells are grown on PPGAS medium, is not as important as that of the other transcription start sites.

It has been proposed that RhlR binds to *lasB* OP2 las box and represses its LasR-dependent expression (2). We found that *rhlR* las box1 is similar to this *lasB* las box (Fig. 5A). It is possible that RhlR exerts its negative auto-regulation by binding to las box1. This possibility remains to be experimentally validated.

Role of the two putative *rhlR* "las box" sequences. The analysis of the sequence of the *rhlR* regulatory region shows two putative las boxes (Fig. 3A). LasR and RhlR activate gene expression by binding to sequences located around 40 nucleotides upstream of the transcription start site (33). Only las box1 is located in the expected position to bind a transcription activator acting on the P1 promoter (Fig 3). As mentioned above, we found that this las box (Fig. 5A) is similar to one (OP2) of the two present in the regulatory region of *P. aeruginosa lasB*. These two las boxes have 11 nucleotides between the invariable CT and AG sequences. It has been shown that *lasB* OP2 las box is a suboptimal LasR/3-o-C12-HSL responsive sequence (2). Our results (Figs. 1, 4 and 6) are compatible with *rhlR* las box1 being the LasR/3-o-C12-HSL recognized sequence for activation of *rhlR* transcription.

The great majority of las boxes, including *rhlR* las box2, present 12 nucleotides between the invariable sequences (32, 33). It has been reported that an A at position 8 and a T on position 13 on canonical las boxes are present on the regulatory regions of genes activated by both regulators (33), even though they might show some specificity towards one of them (2, 6, 22). We found that *rhlR* las box2 does not have these sequence requirements to be recognized by both LasR and RhlR (Fig. 5B).

It has been reported that LasR is sufficient to activate *rhlR* expression in an *E. coli* background (15, 22). We use this heterologous host to determine whether the LasR-

dependent *rhlR* expression was affected by the presence of las box2. The LasR-dependent *rhlR* expression on the *E. coli* strain DH5α background was determined using plasmids pRD58-1 and pRD58-2 (Table 1), both of which have a transcription *rhlR::lacZ* fusion containing the four detected promoters, but the former plasmid lacks the most distal las box2. To do these experiments LasR was expressed from plasmid pMT1 (Table 1) and 3-o-C12-HSL was supplemented. The results obtained (Fig. 6) show that the presence of las box2 has a considerable negative effect on LasR-dependent *rhlR* expression in the *E. coli* background. On the other hand, this result suggests that LasR/3-o-C12-HSL binds to *rhlR* las box2.

Negative regulation by binding of transcription regulators to DNA binding sites that are distant from the promoters (Fig. 3) have not been reported for members of the LuxR family of transcriptional regulators. One of the possible mechanisms for the negative effect of LasR binding to las box2 is the formation of multimers between the LasR molecules bound to this box and LasR molecules attached to las box1 that interfere with transcription initiation or elongation. This hypothesis remains to be experimentally validated.

It can be concluded by the results obtained in this work that *rhlR* is subject to a very complex transcriptional regulation. It presents four transcription start sites, three of which are differentially expressed under different culture conditions. The expression of *rhlR* is LasR dependent when cells are grown on LB medium, but its expression is much less dependent of this quorum-sensing regulator when grown on PPGAS medium. One of the *rhlR* promoters (P3) is σ^{54} dependent. The transcription from some of the *rhlR* promoters is positively regulated by LasR (P1 and P4) and by Vfr (P4) and repressed by RhlR (P2).

The presence of multiple promoters and regulatory sequences in the *rhlR* upstream region suggests that the expression of this gene is tightly regulated by different environmental conditions and that its expression is not only dependent on bacterial density. To our knowledge this is the most complex pattern of transcription regulation described for any of the genes encoding members of the LuxR family of transcription regulators.

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Table 1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Reference
Pseudomonas aeruginosa:		
PAO1	Wild type strain	11
PAOR1	PAO1 *lasR*::Tc mutant	9
PAO9001	PAO1 *vfr*::Gm mutant	25
PG201	Wild type strain	20
65E12	PG201 *rhlR* frameshift mutant	20
PAK	Wild type strain	13
PAKN1	PAK *rpoN*::Tc mutant	13
Escherichia coli:		
DH5α	*sup44, ΔlacU169, hsdR17*, rec*A1, endA1, gyrA96, thi1, relA1*	26
Plasmids:		
pSB1075	*plac-lasR, lasI::luxCDABE*, Ap^r	34
pMP220	vector to construct transcriptional *lacZ* fusions, Tc^r	28
pRD58-1	pMP220 derivative with 288 bp upstream of the *rhlR* ATG codon	This study
pRD58-2	pMP220 derivative with 498 bp upstream of the *rhlR* ATG codon	This study
pPCS1002	contains a *rhlR::lacZ* fusion, Cb^r	23
pUCP20	cloning vector able to replicate in *P. aeruginosa* with *plac*, Cb^r	31
pMT1	pUCP20 derivative with *plac-lasR*	This study

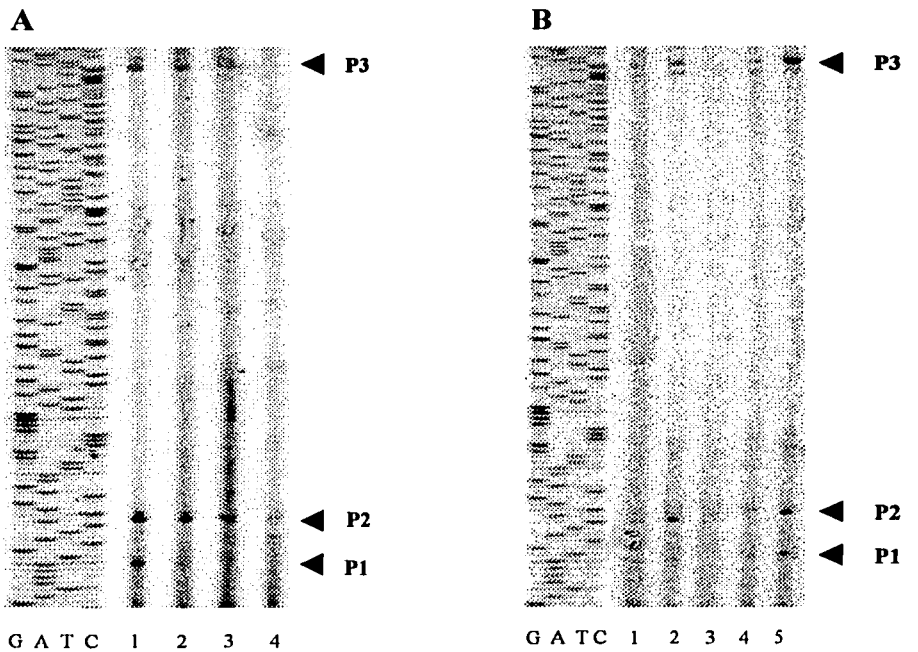


Fig. 1. Primer extension analysis of RNA extracted from different *P. aeruginosa* strains grown on PPGAS medium to an optical density of 1.5 at 600 nm. Oligo-1 was used as primer for the reverse transcriptase reaction and also to determine the DNA sequence (the ladder used to determine this sequence is shown in each panel corresponding to a different polyacrylamide gel). Lanes correspond to RNA extracted from the following strains (A) 1) PAO1 strain, 2) PAOR1, 3) PAK, 4) PAKN1. (B) 1) PG201, 2) 65E12, 3) PAKN1, 4) PAK, 5) PAS1.

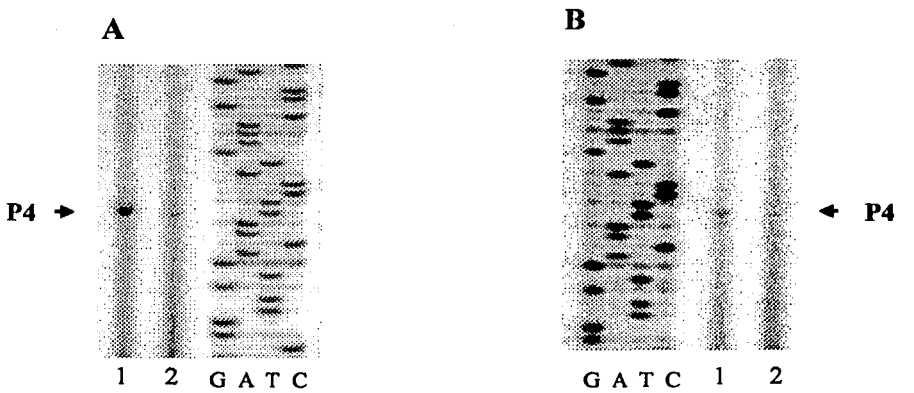
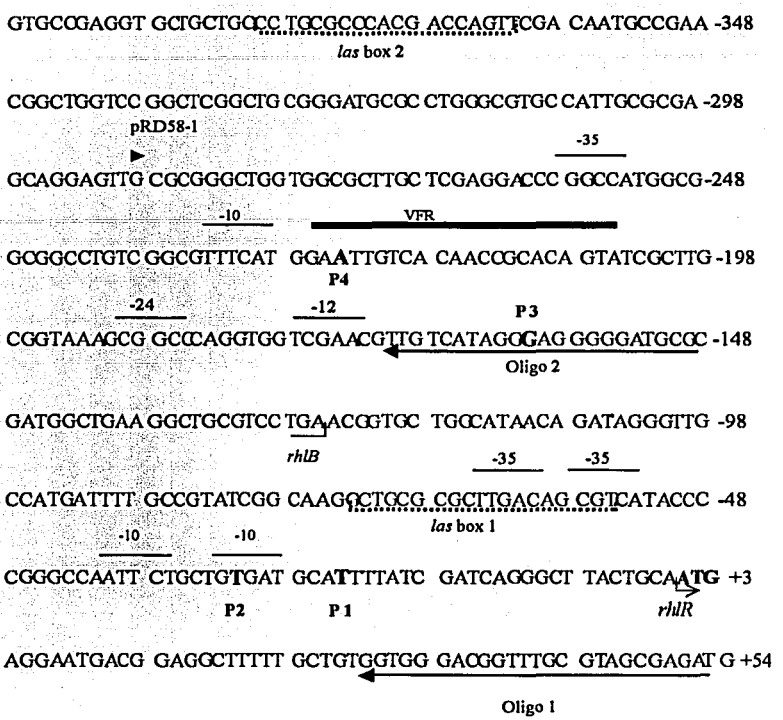


Fig. 2. Primer extension analysis of RNA extracted from PAO1 strain and its *lasR* and *vfr* derived mutants PAOR1 and PAO9001, respectively, grown on PPGAS medium to an optical density of 1.5 at 600 nm. Oligo-2 was used as primer for the reverse transcriptase reaction and also to determine the DNA sequence of the region (the ladder used to determine this sequence is shown). Lanes correspond to RNA extracted from the following strains: (A) 1) PAO1, 2) PAOR1; (B) 1) PAO1 and 2) PAO9001.

A



B

E. coli CRP	A	A	N	T	G	T	G	A	N	N	N	N	N	N	T	C	A	C	A	N	T	T
lasR	A	A	A	T	G	T	G	A	T	C	T	A	G	A	T	C	A	C	A	T	T	T
rhlR	A	A	T	T	G	T	C	A	-	C	A	A	C	C	G	C	A	C	A	G	T	A

Fig. 3. A) Nucleotide sequence of the *rhlR* upstream region showing the four detected transcriptional start sites (bold letters) and their putative -10/-35 or -12/-24 promoter regions. The putative "las boxes" and the Vfr box are also shown. The end of the *rhlB* coding sequence is shown. The sequence underlined corresponds to the oligonucleotides used in the primer extension experiments. The point where the insert in plasmid pRD58-1 starts is shown. B) Alignment of the putaive Vfr box with the Vfr box present in the *lasR* regulatory sequence and with the *Escherichia coli* Crp binding consensus motif.

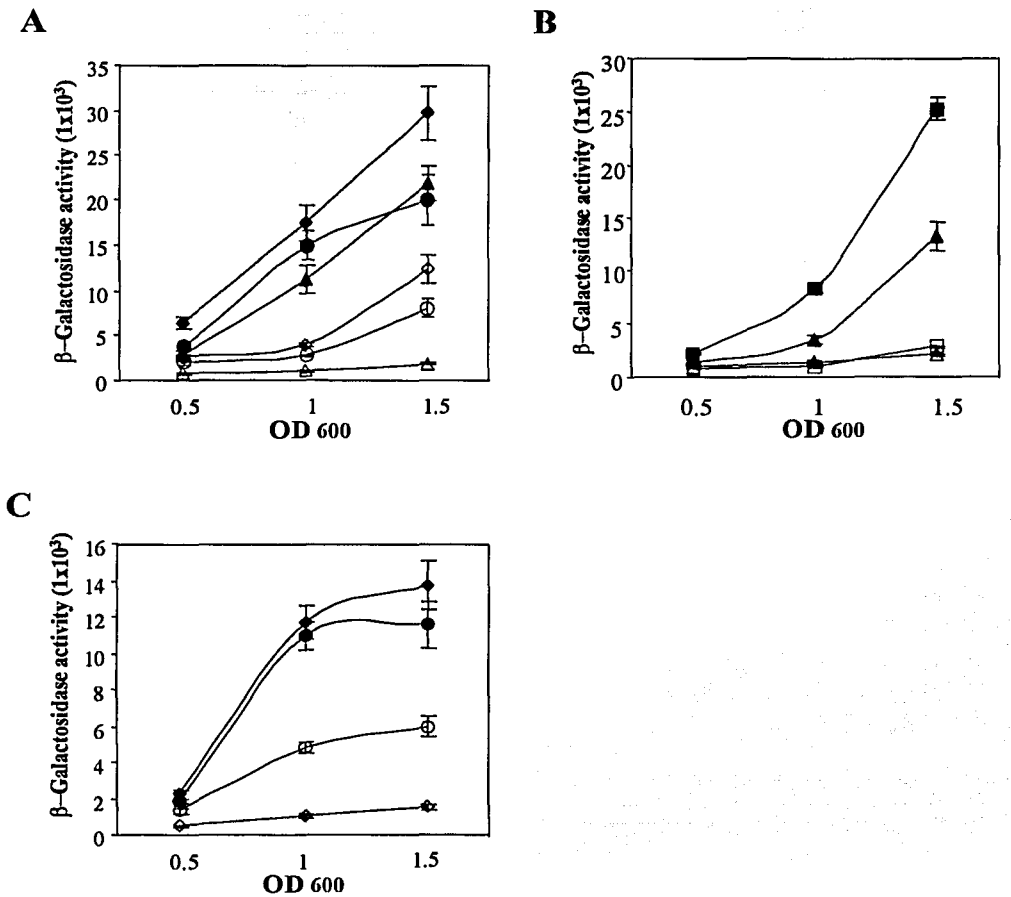


Fig. 4. Expression of the *rhlR::lacZ* fusion encoded in plasmid pPSC1002 along the growth curve of different *P. aeruginosa* strains grown on LB (open symbols) and PPGAS (closed symbols) media. A) Shows strain PAO1 (rhombus), its *lasR* derived mutant PAOR1 (triangles), and its *vfr* derived mutant PAO9001 (circles). B) Presents data obtained with strain PAK (squares) and its *rpoN* derived mutant PAKN1 (triangles) grown on PPGAS medium. C) Shows results obtained with strain PG201 (rhombus) and its *rhlR* derived mutant 65E12 (circles).

A

*rhlR*1	G G C T G C G C G C T - T G A C A G C G
lasB OP2	A C C T G C T T T T C - T G C T A G C T

B

*rhlR*2	C C C T G C G C C C A C G A C C A G T T
lasI	A T C T A T C T C A T T T G C T A G T T
rhlAB	T C C T G T G A A A T C T G G C A G T T
rhlI	C C C T A C C A G A T C T G G C A G G T
lasB OP1	A C C T G C C A G T T C T G G C A G G T
phzA	A C C T A C C A G A T C T T G T A G T T

Fig.5. Alignment of the sequence of the two putative *rhlR* las boxes with las boxes present in the regulatory regions of genes known to be regulated by quorum-sensing regulators. Panel (A) shows the alligment with las box1 and panel (B) with las box2.

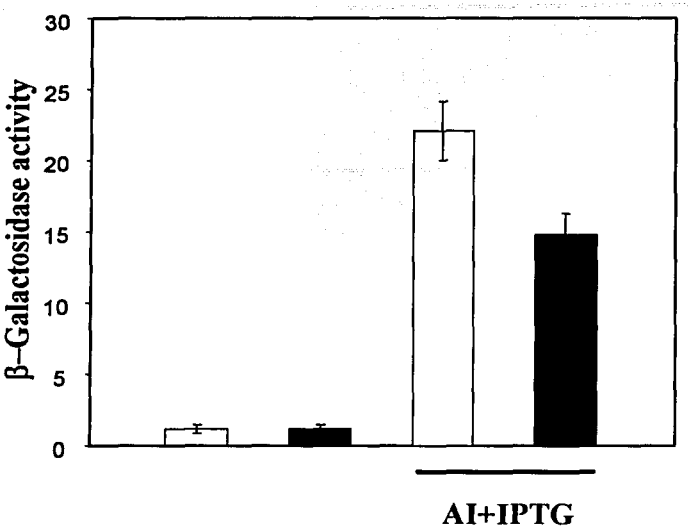


Fig. 6. Expression in *Escherichia coli* of the *rhlR::lacZ fusions* encoded in plasmid pRD58-1 (white bars) which has an insert spanning 288 nucleotides upstream of the *rhlR* translational start site and contains only las box1 and plasmid pRD58-2 (black bars) which contains an insert spanning 498 nucleotides upstream of the *rhlR* translational start site including las box1 and las box2. Expression of the LasR protein encoded in plasmid pMT1 was induced with 0.1 mM IPTG and 3-o-C12-HSL was supplemented at a concentration of 10 μM. β-galactosidase activity is expressed as Miller units.

Resultados III. Artículo sometido a revisión a la revista Molecular Microbiology.

Title: RhlR, a *Pseudomonas aeruginosa* quorum sensing protein, is both an activator and a repressor of the *rhlAB* promoter.

Authors: Gerardo Medina[1], Katy Juárez[1], Brenda Valderrama[2], and Gloria Soberón-Chávez[1]*.

Address: Departamento de Microbiología Molecular[1] and Departamento de Ingeniería Celular y Biocatálisis[2], Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.

*Corresponding author. Mailing address: Departamento de Microbiología, Instituto de Biotecnología, UNAM. Apdo Postal 510-3, Cuernavaca Morelos 62251, México. Phone: (52) (777)3291634. Fax: (52) (777)3172388. E-mail: gloria@ibt.unam.mx

Summary

Pseudomonas aeruginosa contains two transcription regulators--LasR and RhlR--that, when complexed with their specific autoinducers—3-oxo-dodecanoylhomoserine lactone and butanoylhomoserine lactone, respectively--activate transcription of different virulence-associated traits. We studied the RhlR dependent transcription regulation of the *rhlAB* operon encoding rhamnosyltransferase 1, an enzyme involved in the synthesis of the surfactant mono-rhamnolipid, and showed that it represents a dual mechanism of transcriptional regulation. RhlR binds to a specific sequence in the *rhlAB* regulatory region, both in the presence and in the absence of its autoinducer, however, in the former case it activates transcription whereas in the latter it acts as a transcriptional repressor of this promoter.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes serious nosocomial infections. The secretion of numerous toxic compounds and hydrolytic enzymes involved in bacterial pathogenesis are coordinately expressed at high bacterial densities by a mechanism called quorum-sensing (Withers *et al.*, 2001). The quorum-sensing response is triggered by the accumulation in the extracellular medium of certain compounds called autoinducers (AIs). In several proteobacteria the AIs are *N*-acyl-homoserine lactones. At high bacterial densities AIs reach a threshold concentration and interact with specific transcriptional regulators, usually belonging to the LuxR family. These protein-autoinducer (AI) complexes are responsible for the regulation of transcription (Withers *et al.*, 2001).

P. aeruginosa quorum-sensing regulation of gene expression depends on the production of mainly two AIs: butanoyl-homoserine lactone (C4-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-o-C12-HSL) that coupled with RhlR and LasR proteins, respectively, activate gene expression (Fuqua and Greenberg, 1998). The LasR/3-o-C12-HSL complex promotes transcription of several virulence-associated traits including the gene coding for the transcriptional regulator RhlR (Latifi *et al.*, 1996; Pesci *et al.*, 1997). The RhlR/C4-HSL complex promotes the expression, among others, of the genes coding for the enzymes involved in rhamnolipid synthesis, the *rhlAB* operon encoding rhamnosyltransferase 1 (Ochsner *et al.*, 1994) and the *rhlC* gene encoding rhamnosyltransferase 2 (Rahim *et al.*, 2001). Besides the well-documented RhlR ability to activate transcription, it has been suggested that this protein acts as a repressor in at least two different promoters (Medina *et al.*, 2002; Anderson *et al.*, 1999). We have recent evidence suggesting that RhlR can act as a repressor of its own gene *rhlR* (Medina *et al.*, 2002) and it has been suggested that this protein, in the absence of its autoinducer, has a negative effect on the LasR/3-o-C12-HSL-dependent expression of *lasB* (Anderson *et al.*, 1999).

LasR and RhlR activate transcription through binding to a DNA-sequence called las-box, which has been defined by the presence of a conserved sequence and identified in all genes known to be activated by these quorum sensing transcription regulators (Whiteley *et al.*, 1999; Whiteley and Greenberg, 2001). The las-box has a very similar sequence to

that recognized by other members of the LuxR family (Egland and Greenberg, 2000; Whiteley and Greenberg, 2001). Even more, LasR/3-o-C12-HSL complex has been shown to bind and activate the expression of *Vibrio fischeri lux* operon which is normally activated by LuxR/3-oxo-hexanoylhomoserine lactone complex (Gray *et al.*, 1994). The LuxR protein binds preferentially to the lux box when forming a complex with its corresponding AI (Egland and Greenberg, 1994).

Several quorum-sensing transcriptional regulators belonging to the same family as RhlR and LasR have been purified (Lou and Farrand, 1999; Minogue *et al.*, 2002; Nasser *et al.*,1998; Reverchon *et al.*, 1998; Welch *et al.*, 2000; Zhu and Winans, 1999; 2001). Recently, the three dimensional structure of the first member of this family was obtained (Zhang *et al.*, 2002). The TraR activator from *Agrobacterium tumefaciens* was crystallized in complex with its corresponding AI and its target DNA sequence (Zhang *et al.*, 2002). It was found to consist of an asymmetric dimer that interacts with its target DNA sequence through a carboxy-terminal helix-turn-helix motif and with the AI through a hydrophobic pouch present in the amino-half of the protein (Zhang *et al.*, 2002).

It is interesting that some of the LuxR family of regulatory proteins have different mechanisms for transcriptional activation. In the case of *A. tumefaciens* TraR (Lou and Farrand, 1999), *V.fischeri* LuxR (Egland and Greenberg, 2000) and *Erwinia carotovora* CarR (Welch *et al.*, 2000), the proteins bind to its DNA target sequence only when complexed with their corresponding AI. The binding of TraR to its AI has three effects, it increases the binding affinity for its target DNA sequences, it is necessary for this protein to attain an active conformation and prevents degradation by endogenous proteases (Zhu and Winans, 1999; 2001). In the case of CarR it has been shown that the binding of the AI causes its multimerization in the presence of its target DNA sequence (Welch *et al.*, 2000). On the other hand, the *Erwinia chrysanthemi* ExpR regulator can bind to its DNA target sequence even in the absence of its correspondent AI, but its conformation is modified by its binding (Nasser *et al.*, 1998). ExpR functions as a transcriptional activator of some promoters, but it is also a repressor of its own promoter. When ExpR acts as a repressor the presence of AI causes dissociation from its DNA target sequence (Reverchon *et al.*, 1998). *Pantoea stewartii* EsaR and *Serratia marcescens* SpnR bind to

their target sequence in the absence of autoinducer acting as repressors, this interaction is reversed by AI binding (Minogue *et al.*, 2002; Horng *et al.*, 2002).

The aim of this work was to further characterize the mechanism of transcriptional regulation of *P. aeruginosa* RhlR. To achieve this, we studied the heterologous expression of the *P. aeruginosa rhlAB* promoter in *Escherichia coli*. We determined that the RhlR binding DNA sequence corresponds to the predicted las-box in the *rhlAB* upstream region and also that RhlR binds to this DNA sequence, both *in vivo* and *in vitro,* in the presence and in the absence of C4-HSL. The binding of RhlR/C4-HSL to the las-box is a necessary condition for *rhlAB* expression while the binding of RhlR alone to the same DNA sequence represses its transcription. To our knowledge the ability of one protein to activate or repress transcription when bound to the same DNA promoter region depending on coligand binding, as is shown here for RhlR, has not been reported previously for any other bacterial transcriptional regulator.

Results

Transcription of rhlAB *in* Escherichia coli *is* σ^{54}*-independent*

The transcription start site of the *Pseudomonas aeruginosa rhlAB* operon has been reported (Pearson *et al.*,1997). To further characterize this promoter we evaluated its functionality in *Escherichia coli* . We determined that the *rhlAB* transcription start site in *E. coli* coincided with the previously reported site for *P. aeruginosa* (Fig. 1). The *rhlAB* regulatory region contains canonical –10 and –35 corresponding to a promoter transcribed by RNA-polymerase (RNA-P) containing a σ^{70}-subunit (Pearson *et al.*, 1997). However, the transcription of this promoter has been proposed to be dependent on the RNA-P containing the σ^{54} subunit, based on the reduced level of its expression on a *P. aeruginosa rpoN* mutant (Pearson *et al.*, 1997). We analyzed the role of σ^{54} in *rhlAB* expression in *E. coli* by determining its level of transcription and start site in a mutant devoid of this sigma factor. Figure 1 shows that the expression of this promoter is independent of σ^{54}. In support of these results the RhlR transcriptional regulator does not have sequence similarity to the EBP family of transcriptional activators like NtrC and NifA, that are specific for expression of σ^{54}-dependent promoters (González *et al.*, 1998).

Thus RhlR cannot activate a σ^{54}-dependent promoter and the data presented in this work are related to the transcriptional regulation of *rhlAB* at a σ^{70}-dependent promoter.

RhlR specifically binds to the rhlAB *"las box" with or without C4-HSL*

The *rhlAB* upstream region contains a single las-box whch is centered at –42 nucleotides with respect to the transcriptional start site (Pearson *et al.,* 1997). To study the specific binding of the RhlR protein *in vitro,* gel retardation assays were performed using a 216 bp DNA fragment corresponding to *rhlAB* upstream region including the las-box, with an *E. coli* DH5α/pECP61.5 (*ptac-rhlR* and *rhlA::lacZ*) cell-free extract over-expressing RhlR (approximately 5% of total cell protein, data not shown). Retardation of the labeled DNA fragment depended on the presence of plasmid pECP61.5 and on the use of a DNA fragment which contains the las-box sequence (Fig. 2). Interestingly, we found that RhlR binds to its target sequence both in the presence and absence of its AI, C4-HSL (Fig. 2). This is in contrast to many other transcriptional regulators of the LuxR family which bind to DNA only in one of these conditions (Egland and Greenberg 2001; Gray *et al.,* 1994; Lou and Farrand, 1999; Nasser *et al.,* 1998; Welch *et al.,* 2000). The addition of 3-o-C12-HSL alone or with C4-HSL has no effect on RhlR binding to its target DNA (data not shown).

Our results (Fig. 2C) suggest that the binding affinity of RhlR to its target DNA sequence is slightly higher than that of RhlR/C4-HSL. The same cell-free extract expressing RhlR presented a smaller proportion of DNA retarded in the gels when C4-HSL is added to the reaction mixture (Fig. 2C). Similar results were obtained even when the cell-free extract was incubated with AI overnight (data not shown). However, to determine RhlR and RhlR/C4-HSL dissociation constant to its target DNA it will be necessary to use purified RhlR.

Gel retardation assays only show whether a protein interacts with a specific DNA fragment, but more precise information, as what type of complex is formed, or what is the DNA sequence involved in this interaction cannot be obtained by these type of experiments. To determine whether the conformation of the RhlR/las-box complex was modified by the interaction of RhlR with its autoinducer we used an *in vivo* DNA methylation protection assay (Fig. 3). To do this experiment we used two plasmids pGMYC (*plac-rhlR*) and pMPCG (*rhlA::lacZ*). In agreement with the results obtained in

the gel retardation assay, we found that RhlR binds *in vivo,* both in the presence and absence of C4-HSL, to a DNA region that includes the sequence defined as the las-box, since several guanine residues in this region were protected from methylation or hypermethylated (Fig. 3). However, the *in vivo* footprinting assay enabled us to detect several differences in the way that RhlR binds to its target DNA sequence depending on its coupling to C4-HSL (Fig. 3). Changes in the methylation of guanine residues, either protection or hyper-methylation, were apparent in the sequence spanning from guanine at position –20 to that at –90 with respect to the *rhlAB* transcription start site. The different conformation of the DNA-bound RhlR protein, with or without AI, is apparent by the differential protection of the guanine residues at –20, -39, -54, -64 and -78. The protection of guanines at –39 and –54, and hyper-methylation of those at positions –20, -64 and -78 by RhlR binding are only apparent in the presence of RhlR without AI (Fig. 3).

RhlR uncoupled to C4-HSL is a repressor of rhlAB *transcription*

As was previously described (Pearson *et al.,* 1997) we found that RhlR/C4-HSL is necessary for *rhlAB* transcriptional activation (Table 2). No expression of the *rhlA::lacZ* fusion was seen in the absence of C4-HSL (Table 2). The LasR/3-o-C12-HSL complex can activate *rhlAB* promoter expression, but at a considerably lower level than the RhlR/C4-HSL complex (Pearson *et al.,* 1997 Table 2). Unexpectedly, however, we found that the expression of *rhlAB* promoter is slightly, but reproducibly, lower when RhlR expression is induced by the addition of 1 mM IPTG, even in the presence of C4-HSL (Table 2). This result suggests that RhlR unbound to its AI could act as *rhlAB* transcriptional repressor. The over-expression of LasR by IPTG addition had no repressor activity on *rhlAB* promoter expression (Table 2).

To further investigate whether RhlR can act as a repressor of the *rhlAB* promoter we used plasmid pECP61.5 that contains *rhlR* under the control of the *tac* promoter and a *rhlA::lacZ* fusion in the same plasmid. The use of a plasmid contaning both the gene coding for the transcriptional regulator and the gene that is regulated minimizes the possibility of artifacts due to different copy numbers of the plasmids used. Using this system we measured *rhlA::lacZ* expression and RhlR concentration in the cell soluble fraction by Western immunobloting on DH5α/pECP61.5 cells grown in the presence of 1 mM C4-HSL with and without 1 mM IPTG (Fig. 4). Under these conditions it was

apparent that *rhlA::lacZ* is considerably repressed (up to approximately 33%) correlating with an accumulation of RhlR (Fig. 4).

RhlR repressor activity is not specific on its expression in the *E. coli* background, since it is also detected in *P. aeruginosa* PAO1 background. Figure 5 shows that increasing RhlR concentration by adding 1 mM IPTG to a *P. aeruginosa* PAO1/pECP61.5 culture resulted in a reduced level of *rhlAB* expression (attaining almost 50% of repression).

The low level of *rhlAB* repression seen using plasmids pGMYC (*plac-rhlR*) and pMPCG (*rhlA::lacZ*) (Table 2) might be explained by the small difference in *rhlR* expression under the conditions of repression and induction used in these experiment. β-galactosidase activity expressed by DH5α/pUCP20 (the vector used to construct plasmid pGMYC) in the absence of induction is 118.1±4.0 and with 1 mM IPTG is 193.0±1.4. These results show that expression of *plac* is leaky and that full induction of this promoter only accounts for a 63% increase in the enzyme activity detected.

The repression of *rhlAB* promoter by RhlR can also be visualized by adding IPTG and thus increasing RhlR concentration, to an *E. coli* DH5α/pMPCG-pGMYC culture after 4 h of growth in the presence of AI (data not shown). Repression of *rhlAB* transcription by RhlR is a reversible phenomena since addition of C4-HSL to an *E. coli* DH5α/pMPCG-pGMYC culture after 4 h of growth in the presence or absence of 1 mM IPTG results in *rhlA::lacZ* induction, although the final level of expression is lower in the latter case (data not shown).

Discussion

In this work we have shown that *rhlAB* promoter is not dependent on σ^{54} for its expression in *Escherichia coli* (Fig. 1). It is very much likely that the RhlR/C4-HSL-dependent expression of this promoter in *Pseudomonas aeruginosa* is also independent of this sigma factor, since this protein does not belong to the EBP family (Morett and Segovia, 1993). Thus, the observed dependence of *rhlAB* expression on a functional σ^{54} (Pearson *et al.*, 1997) seems to be due to an indirect effect. We have recently reported (Medina *et al.*, 2002) that *rhlR* has four different transcriptional start sites and that one of

these is σ^{54}-dependent. This could explain, at least in part, the apparent regulation of *P.aeruginosa rhlAB* expression by this alternative sigma factor.

Bacterial transcriptional activators of promoters recognized by RNA-P containing the σ^{70} subunit have been thoroughly characterized (Rhodius and Busby, 1998). Those activators that have binding sites centered at –40 and thus overlapping the –35 promoter sequence have been found to make direct contact with region 4 of the σ^{70} subunit and with the carboxy-terminal domain of the α subunit of RNA-P (Rhodius and Busby, 1998). These activators "recruit" RNA-P to the promoter by increasing its apparent affinity for the –35 sequence. Since RhlR binds at –40 of the *rhlAB* transcriptional start site (Fig. 3), we proposed that this is the mechanism of *rhlAB* transcription activation by RhlR/C4-HSL, as has been shown for LuxR that is the most studied member of this family of regulatory proteins (Stevens *et al.*, 1999).

There are transcription repressors that bind to an upstream region of the –35 promoter sequence and make contact with RNA-P, preventing it to proceed with gene transcription, either at the level of initiation or elongation (Rojo, 2001). Our data show that RhlR unbound to C4-HSL binds to its target DNA (Figs. 2 and 3) and that in this condition it represses *rhlAB* transcription (Table 2, Figs. 4 and 5).

Expression of *rhlAB* promoter is strictly dependent on the presence of RhlR and C4-HSL (Fig. 1, Table 2). However, RhlR binds *rhlAB* las-box with a slightly higher affinity (Fig. 2C), thus the unbound regulatory protein will act as an anti-activator by competing with the proportion of RhlR/C4-HSL for las-box binding. This dual transcriptional regulatory mechanism permits a very sensitive and rapid control of gene expression depending on the available AI concentration. To our knowledge, this is the first report of a bacterial regulatory protein which, by binding to a single target sequence, activates or represses the same promoter, depending on its interaction with a metabolic effector. The DNA methylation protection assay showed that the DNA (las-box) complexes with RhlR and with RhlR/C4-HSL are different (Fig. 3). We found that the protection of guanines at –39 and –54, and hyper-methylation of those at positions –20, -64 and -78 by RhlR binding are only apparent with free RhlR (Fig. 3). Figure 6 shows a diagrammatic representation of RhlR binding to the *rhlAB* promoter region with and without C4-HSL, in the former case transcriptional activation is attained, while in the latter it is repressed.

RhlR can act as a repressor by blocking RNA-P binding to the *rhlAB* promoter or transcriptional elongation. One possibility to explain our results is that the binding conformation of RhlR unbound to C4-HSL interferes with RNA-P binding to the –35 sequence. This possibility is supported by the differential DNA interaction of RhlR and RhlR/C4-HSL with the –35 *rhlAB* promoter region (Fig. 3). An alternative explanation for RhlR repressing activity is that this regulatory protein makes contact with RNA-P and that this interaction prevents transcriptional elongation. A more detailed analysis of this regulatory protein is necessary to rule out one of these possible mechanisms.

Transcription of *rhlAB* operon is fully activated by RhlR/C4-HSL, but LasR/3-o-C12-HSL can activate its expression at a lower level (Table 2). We found that increasing LasR expression did not resulted in repression of *rhlAB* expression (Table 2). There are other genes in *P. aeruginosa*, like *lasB,* that are regulated by these proteins in an opposite manner: they are fully activated by LasR/3-o-C12-HSL and to a lesser extent by RhlR/C4-HSL (Pearson *et al.,* 1997). It has been proposed, based on the regulation of *lasB* promoter in different *P. aeruginosa* mutants that RhlR when unbound to C4-HSL acts as a repressor of this promoter (Anderson *et al.,* 1999). This previous report thus provides experimental evidence with a different promoter (*lasB*), in support of the model presented in this work for RhlR regulation of transcription of the *rhlA* promoter (Fig. 6). Even more, we have evidence suggesting that RhlR represses the expression of its own gene (Medina *et al.,* 2002).

The dual function of RhlR as an activator and repressor of gene transcription represents a new element in the *P. aeruginosa* quorum-sensing response that needs to be taken into account to understand this complex and fine-tuned genetic regulatory network.

Experimental procedures

Bacterial strains, plasmids, and culture conditions

Escherichia coli strains DH5α (Sambrook *et al.,* 1989) or ET8000 (McNeil *et al.,* 1982), cultured in Luria Bertani (LB) broth (Miller, 1972), were used as stated. *Pseudomonas aeruginosa* PAO1 (Hancock and Carey, 1979) was grown in phosphate-limited peptone-glucose-ammonium salts medium (PPGAS; pH 7.2) containing NH_4Cl_2 (0.02 M), KCl (0.02 M), Tris-HCl (0.12 M), $MgSO_4$ (0.0016 M), glucose (0.5%), and peptone (1%) (Zhang and Miller, 1992). Plasmids used are listed in Table 1. When appropriate, isopropyl-

β-D-thiogalactoside (IPTG) was added to the medium at the concentration stated in the text. Antibiotics (Sigma Chemical Co.) were used to supplement selection media at the following concentrations in μg/ml for *E. coli* and *P. aeruginosa*, respectively: ampicillin 150 and not used; carbenicillin not used and 250; tetracycline 15 and 100. *N*-acyl-homoserine lactones (C4-HSL and 3-o-C12-HSL) were purchased from Quorum Sciences Inc. (Coralville, IA). These AIs were used at a concentration of 10 μM, unless otherwise stated. β-galactosidase activity was determined as reported (Miller 1972). One Miller Unit corresponds to 1 nM of o-nitrophenyl-b-D-galactopyranoside hydrolyzed per minute and per absorbance unit at 600 nm, the data shown in this work represent the average of triplicate measurements from two independent experiments.

Strain ET8000*rpoN::Tn*5 was constructed by transducing the *rpoN::Tn*5 mutation from strain MX848 (Osorio *et al.*, 1984) to strain ET8000 using phage P1cm1,clr100, as described elsewhere (Miller 1972).

Enzymes and reagents

Plasmids were purified using the Wizard plus minipreps DNA purification system (Promega corporation Madison WI). DNA bands were cut from agarose gels and purified with Geneclean III kit (Bio101 La Jolla CA). DNA restriction enzymes were purchased from New England Biolabs (Beverly Mass.) or Roche Diagnostics Corporation (Indianapolis IND), and used according with the manufacturers' instructions.

Nucleic acid techniques

DNA manipulation was performed as reported by Sambrook *et al.*, 1989. DNA sequencing reactions were done using the Thermosequenase kit (Amersham Life Science Corp. Cleveland, Ohio). Oligonucleotides were radioactively labeled with the T4 polynucleotide kinase (Amersham Life Science Corp. Cleveland, Ohio), using [γ-^{32}P]-ATP (Amersham International) as substrate. Blunt ended DNA fragments were obtained with the Klenow fragment of DNA polymerase I (Amersham Life Science Corp. Cleveland, Ohio).

Plasmid pMPCG was constructed by ligating the 0.8 kb *Hind*III-*Bam*HI fragment from plasmid pUO58 (Ochsner *et al.*, 1994) containing the *rhlAB* promoter into the same restriction sites of plasmid pMP220 (Spaink *et al.*, 1987) to create a *rhlA::lacZ* transcriptional fusion. Plasmid pGMYC (Medina *et al.*, 2002) was constructed by ligating a 1.3kb blunt-ended *Sma*I-*Kpn*I fragment from pUO58 (Ochsner *et al.*, 1994) unto pMOS-Blue and then subcloning *rhlR* into the *Sma*I site of pUCP20 (West *et al.*, 1994) to render *rhlR* under *plac* control.

Primer extension analysis

E. coli strains containing plasmid pECP61.5 (Pearson *et al.*, 1997) were grown to an optical density of 1.5 at 600 nm and their total RNA was extracted using a GlassMAX RNA Microisolation Reagent Assembly (Life technologies Inc. Gaithesburg, MD). Primer extension reactions were performed using the RAV2 reverse transcriptase (Amersham Life Science Corp. Cleveland, Ohio) according to the manufacturers' instructions. All reactions were carried out at 42^{0}C. The experiments were done using a primer (oligo1) that corresponds to the sequence between -159 to -179 with respect to the *rhlA* ATG codon: 5'-GGGGCTTGTGTGGGTCTTGC-3'.

Gel retardation assays

A 216 bp radiolabeled DNA fragment containing the *rhlAB* regulatory region was synthesized by PCR, using plasmid pECP61.5 as template and oligo1 and oligo2 (5'-CATGCCTTTTCCGCCAACCCCTCGC-3') as primers. One of the primers used to amplify this fragment was radiolabeled with [γ-^{32}P]-ATP (Amersham International) prior to the PCR reaction, using T4 polynucleotide kinase. Binding reactions were carried out in the following buffer: 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 60 mM KCl (Fig. 2A and 2B) or 100 mM KCl (Fig. 2C) and 10% glycerol. Calf thymus DNA (300 μg/ml) was included in all reactions. After 20 min of incubation at room temperature (Fig. 2A and 2B) or 37^{0}C (Fig 2C), samples were size fractionated on 6% polyacrylamide gels in 0.5X TBE buffer (Sambrook *et al.*, 1989).

Under conditions of *ptac* induction by IPTG, a considerable fraction of RhlR is present in inclusion bodies. This insoluble protein does not bind to the DNA fragment containing the las-box (data not shown).

In vivo methylation protection assays

In vivo methylation protection assays of the *rhlAB* operon by binding of the RhlR protein in *E. coli* were performed as described (González, *et al.*, 1998) with the following modifications: *E. coli* DH5α cells with the plasmids pMPCG and pGMYC were inoculated in LB medium until stationary phase. Dimethyl sulfate (DMS) was added at a final concentration of 0.1% and cells were incubated for 1 min. The cell pellet was washed twice with 50 ml of ice-cold saline phosphate solution. Plasmid DNA was purified and cleaved at the methylated positions by incubation with 10% piperidine at 90^{0}C for 30 min. Piperidine was vacuum evaporated and plasmid DNA was washed twice with 70% ethanol. Primer extension reactions were performed using the purified plasmid DNA as template and either end-labeled oligo1 or oligo2 as primers for each DNA strand. The concentrated extension products were separated by electrophoresis in a 7% polyacrylamide gel containing 8 M urea. The results shown in figure 2 correspond to one of the *rhlA* DNA strands, but we found that the same DNA sequence was protected when we analyzed the other strand using oligo2 (data not shown). Determination of DNA sequences was done with the same primers.

Overproduction of the thioredoxin (Thio)-RhlR protein fusion

To construct a Thio-RhlR protein fusion, the *rhlR* gene was PCR amplified using oligo3 5'-GAGACTGCAGGTCGACTCAGATGAGGCCCAG-3' and oligo4 5'-GATAGGTACCAGAATTCATGAGGAATGACGGA-3'. The product was digested with *Kpn*I and *Sal*I and cloned into plasmid pThioC (invitrogen) digested with the same enzymes, the resulting plasmid pThio-R was transformed into *E. coli* DH5α. This strain was cultured at 30^{0}C and when induced with 0.5 mM IPTG most of the expressed fusion protein formed inclusion-bodies. Cells were harvested by centrifugation and ruptured by sonication. The pellet obtained after centrifugation of cell lysate was resuspended and size-fractionated by SDS/PAGE. The band corresponding to Thio-RhlR protein was cut, crushed and used to immunize rabbits.

Immunobloting techniques

New Zealand rabbits were immunized with the protein fusion Thio-RhlR in order to produce polyclonal antibodies. *E. coli* cultures were grown until they reached the indicated optical density at 600 nm. The cells

were collected and lysed by boiling for 5 min in loading buffer (Sambrook *et al.*, 1989). Equal amounts of protein from each lysate were separated in a 14% SDS/PAGE. The proteins were transferred by electroblotting from the gel to Hybond-C nitrocellulose membranes (Amersham Life Science Corp.). RhlR was detected by using the rabbit polyclonal antiserum raised against ThioR-RhlR.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pECP61.5	contains an *rhlA::lacZ* translational fusion and *rhlR* under ptac, Ap^r	Pearson *et al.*, 1997
pMP220	vector to construct transcriptional *lacZ* fusions, Tc^r	Spaink *et al.*, 1987
pMPCG	pMP220 derivative with an *rhlA::lacZ* fusion	This study
pUCP20	cloning vector with plac, Ap^r	West *et al.*, 1994
pGMYC	pUCP20 expressing *rhlR* under plac	Medina *et al.*,*2002*
pMT1	pUCP20 expressing *lasR* under plac	Medina *et al.*,*2002*
pThio-R	plasmid expressing a thioreductase RhlR fusion-protein, Ap^r	This study

Table 2. Expression of an *rhlA::lacZ* fusion in *Escherichia coli* DH5α β-galactosidase actvity in Miller Units (% of maximun activity)[a]

Plasmid	NA[a]	AI[a]	IPTG[a]	AI + IPTG
pMCG	2.3±0.7 (1.4)	4.1± 0.3 (2.6)	1.9±0.4(1.2)	1.9± 0.4 (1.2)
pMCG/pGMYC	1.5±0.2 (0.9)	157 ±14 (100)	1.2±0.1 (0.7)	130 ±12 (83)
pMCG/pMT1	0.6±0.2 (0.3)	12.3±1.3 (7.8)	0.6±0.3 (0.3)	12.1±0.4 (7.6)

[a](%) refers to the percentage of the maximum detected activity in all experiments presented in the table. NA means no addition. AI refers to C4-HSL (experiments shown on the first two lines) or 3-o-C12-HSL (experiments shown on the third line) added at 10 μM. IPTG was added at 1 mM. All measurements were done after 8 h of growth on LB medium.

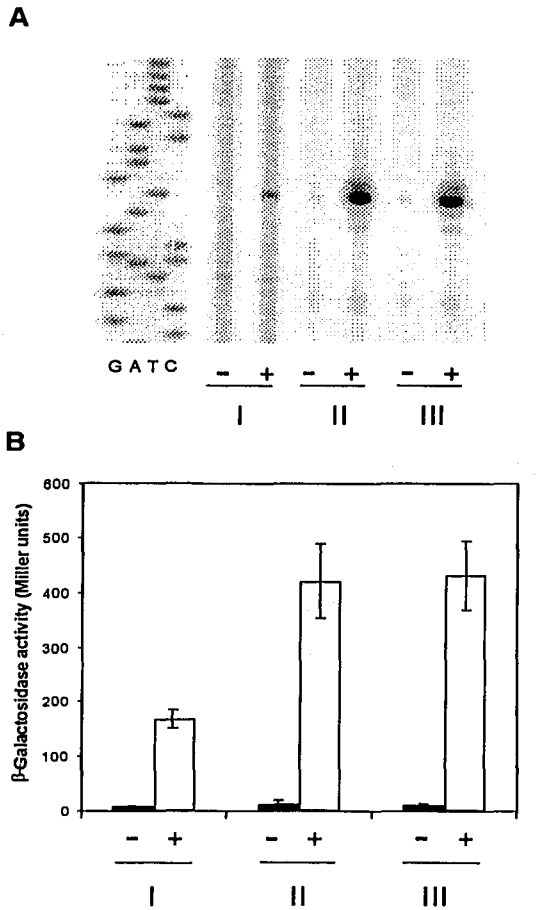

Fig. 1. Identification of *rhlAB* transcription start site by primer extension analysis (A), and analysis of the level of expression (B) in different *E. coli* strains. Panels show the results with the following strains: I) DH5α/pECP61.5; II) ET8000/pECP61.5 and III) ET8000*rpoN::Tn5*/pECP61.5. The presence (+) or absence (-) of 10 μM C4-HSL is indicated.

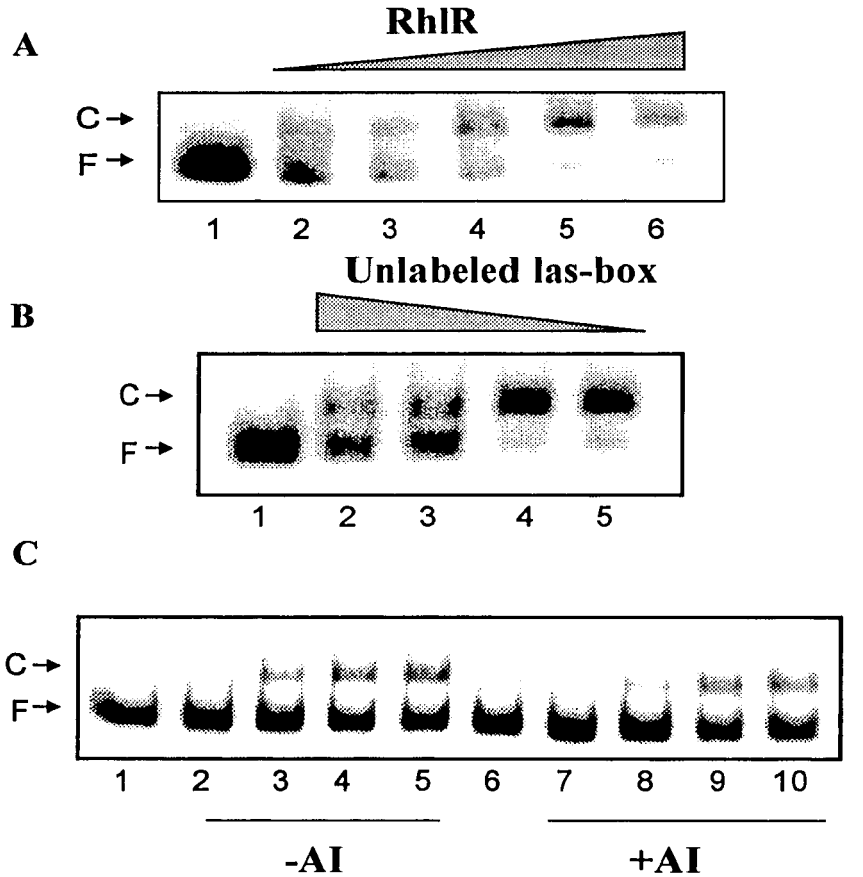

Fig. 2. Gel retardation assays of a 216 bp end-labeled PCR product of the *rhlA* 5' region in the presence of *E. coli* DH5α/pECP61.5 culture cell-free extract induced with 0.5 mM IPTG. A) Increasing amounts of RhlR without AI were used on these binding reactions (lanes 2-6) or without protein (lane 1). B) Competition assay using increasing amounts of a non-labeled PCR product containing *rhlAB* regulatory region. C) Effect of C4-HSL binding to RhlR on its DNA binding ability. Lanes show increasing amounts of cell free extract expressing RhlR (lanes 2-5) and the same cell-free extracts in the presence of 6 mM C4-HSL (lanes 7-10), (C4-HSL was added 5 min before the binding reaction), cell-free extract of *E. coli* DH5α (lane 6) and without protein (lane 1). Bands corresponding to RhlR-DNA complex (C) and free DNA (F) are shown.

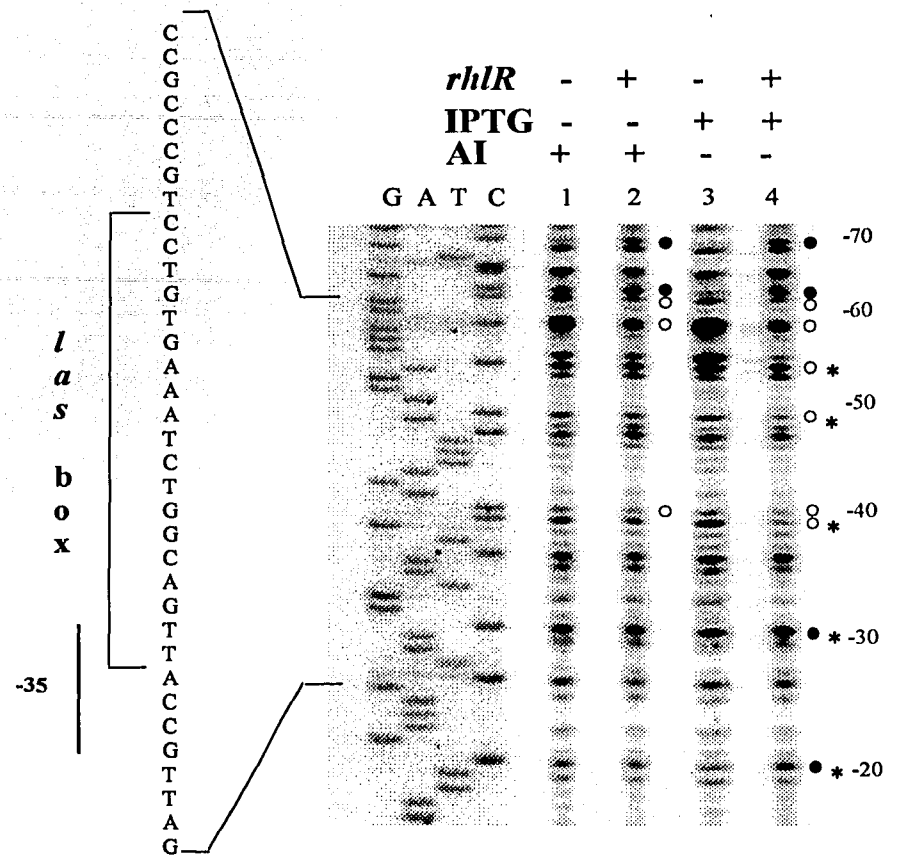

Fig. 3. Dimethyl sulfate footprinting analysis of the RhlR binding to the 5' *rhlAB* region. Lanes correspond to: 1) *E. coli* DH5α/pMPCG,pUCP20 supplemented with C4-HSL, 2) *E. coli* DH5α/pMPCG,pGMYC supplemented with C4-HSL, 3) *E. coli* DH5α/pMPCG,pUCP20 supplemented with 1 mM IPTG, and 4)) *E. coli* DH5α/pMPCG,pGMYC supplemented with 1 mM IPTG. Oligo-1 was used as primer for the reverse transcriptase reaction and also to determine the DNA sequence (the ladder used to determine this sequence is shown). The sequence and position of the predicted las-box and of the -35 promoter sequences are shown at the left of the figure. The position of the protected (open circles) or hypermethylated (closed circles) guanine residues are shown, those having an * are only protected or hypermethylated in the absence of C4-HSL and in the presence of IPTG (lane 4).

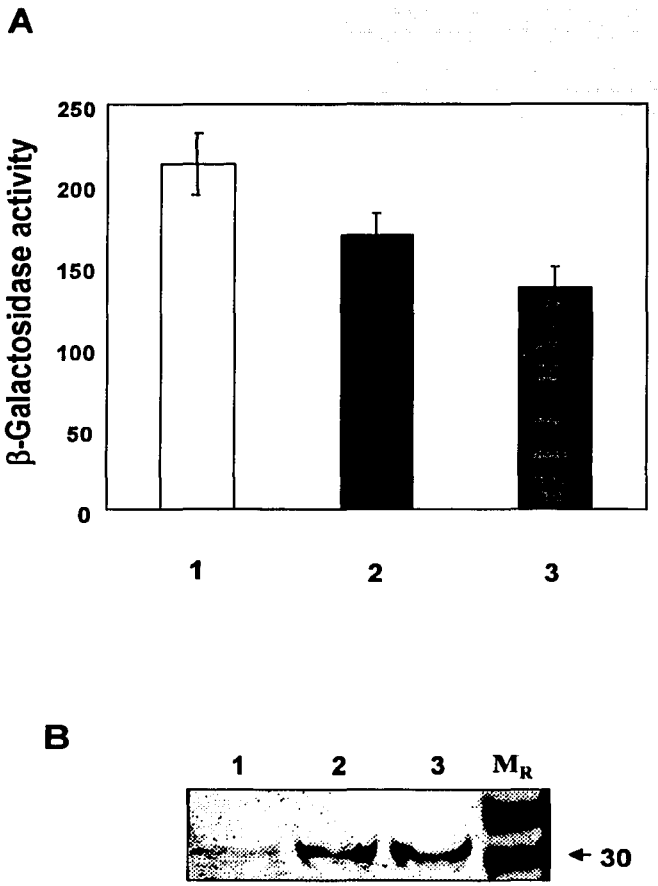

Fig. 4. β-galactosidase activity (A) and RhlR concentration detected by immunobloting in the cell-free extract supernatant (B), after 8 h of growth of *E. coli* DH5α/pECP61.5 in the presence of 10 μM C4-HSL and the following IPTG concentrations: 0, white bar (1A) and lane 1B; 0.5 mM, black bar (2A) and lane 2B; 1 mM, hatched bar (3A) and lane 3B. The molecular size markers are shown with an arrow pointing the 30 kD standard.

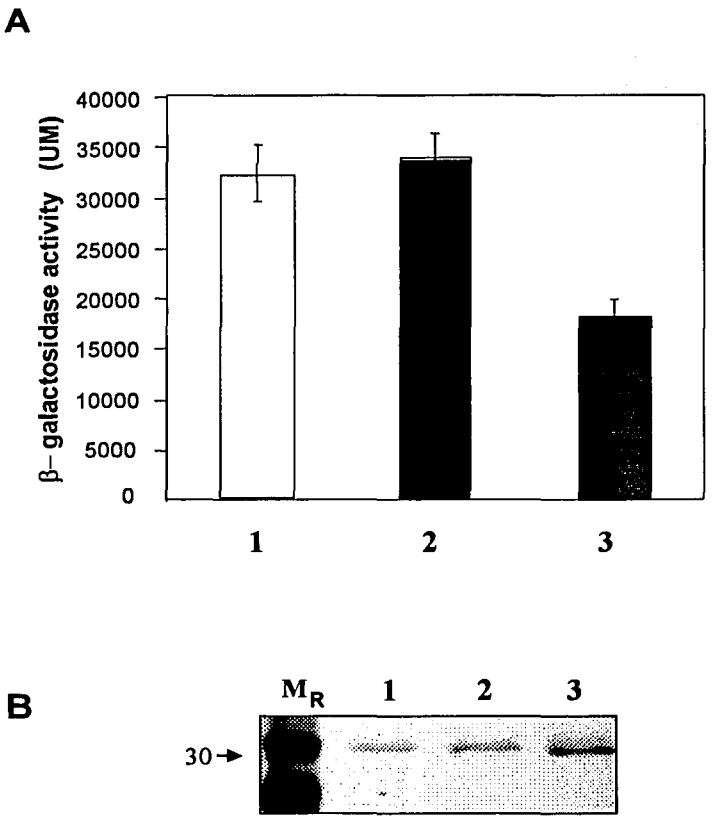

Fig. 5. β-galactosidase activity (A) and RhlR concentration detected by immunobloting (B), after 12 h of growth of *P. aeruginosa* PAO1/pECP61.5. Lanes correspond to: 1) unsupplemented culture medium, 2) addition of 10 μM C4-HSL, and 3) addition of 10 μM C4-HSL and 1 mM IPTG. M_R refers to molecular weight markers.

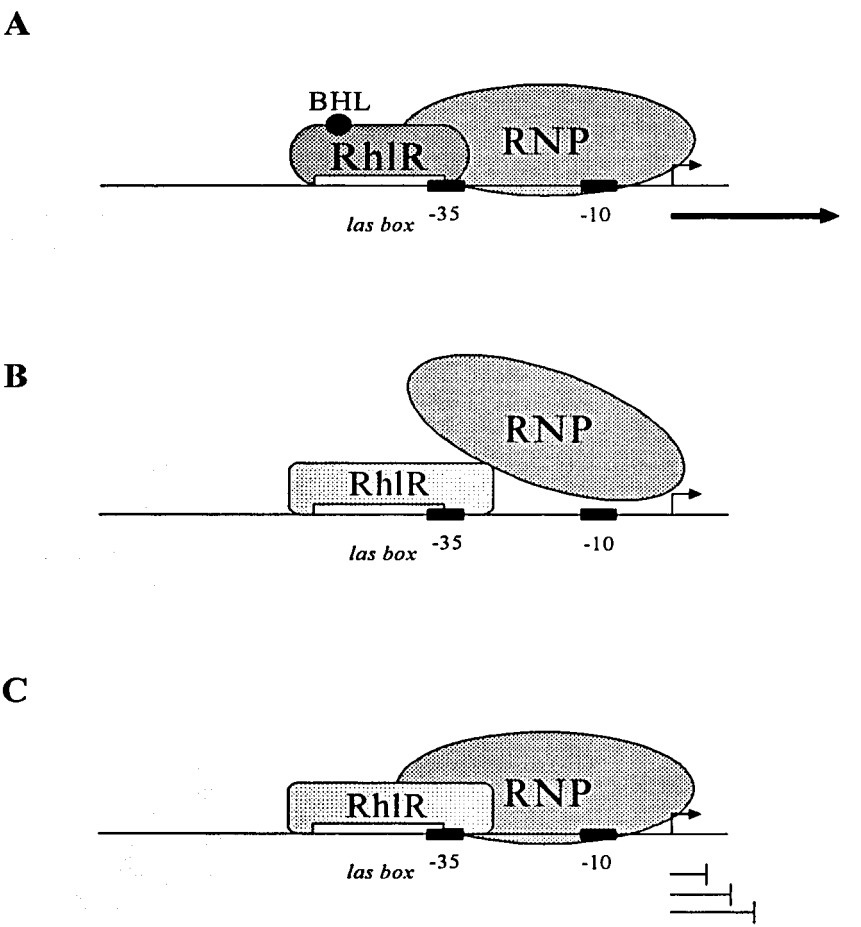

Fig. 6. Schematic representation of the proposed model for RhlR transcriptional regulation of the *rhlA* promoter.

Resultados y discusiones adicionales

Expresión de *rhlR* y producción de ramnolípidos en la cepa PAO1 de *P. aeruginosa* en diferentes medios de cultivo.

Para determinar si la biosíntesis de ramnolípidos depende además de a la densidad celular de la composición del medio de cultivo, se midió la expresión de una fusión transcripcional *rhlR::lacZ* contenida en el plásmido pPCS1002 (Pesci et. al., 1997), con el cual se transformó la cepa PAO1 de *P. aeruginosa* y se midió la concentración de ramnolípidos por el método de orcinol (Zhang y Miller, 1992). Se creció la cepa en diferentes medios de cultivo a 37°C a una densidad óptica a 600 nm de 1.5 correspondiente a fase estacionaria temprana, ó después de 24 horas de crecimiento cuando se encuentra en fase estacionaria tardía y con una densidad de aproximadamente de 2.5 de absorbancia a 600nm. Los resultados obtenidos muestran que la producción de ramnolípidos y la expresión de *rhlR* aumentan bajo condiciones de limitación de nutrientes y el incremento en la producción de ramnolípidos correlaciona siempre con un incremento en la expresión del gen regulador, sin embargo la expresión de *rhlR* no es suficiente para que ocurra una alta producción de ramnolípidos ya que en algunas condiciones se observó una alta expresión de *rhlR* y nula o poca producción de ramnolípidos.

A continuación se describen algunos puntos respecto a los distintos medios utilizados: El efecto de la fuente de carbono fue determinado creciendo la cepa PAO1/pPCS1002 en medio M9 con gluconato, glucosa o glicerol como única fuente de carbono. El gluconato es una fuente de carbono preferida sobre glucosa o glicerol (Lessie y Phibbs, 1984), en tanto el glicerol es la menos preferida como se evidenció por el lento crecimiento de *P. aeruginosa* en dicha fuente de carbono. Se observó una mayor producción de ramnolípidos cuando se utilizó glicerol en vez de glucosa o gluconato y la expresión de *rhlR* también fue la más alta (Figura 8). Lo anterior parece ser debido a que *P. aeruginosa* detecta la disponibilidad de nutrientes y enciende o apaga distintos grupos de genes necesarios para vivir en tales condiciones, de tal manera que cuando crece en glicerol como única fuente de carbono se enciende el sistema detector de quórum como parte de una respuesta a la escasez de nutrientes produciéndose ramnolípidos, los cuales hacen mas accesibles algunas fuentes de carbono alternativas (Zhang y Miller, 1995). De manera interesante la expresión de *rhlR* fue alta al utilizar gluconato como fuente de carbono sin

embargo la producción de ramnolípidos fue pobre (Figura 8). Desconocemos el nivel regulatorio que determina la baja producción de ramnolípidos en esta condición. En la misma figura se observa que cuando se aumenta diez veces la concentración de amonio en el medio M9 utilizando glucosa como fuente de carbono (cambio de la relación carbono/nitrógeno), ocurre una baja expresión de *rhlR* y una pobre producción de ramnolípidos, lo cual sugiere que existe una relación entre el metabolismo de nitrógeno y el sistema detector de quórum.

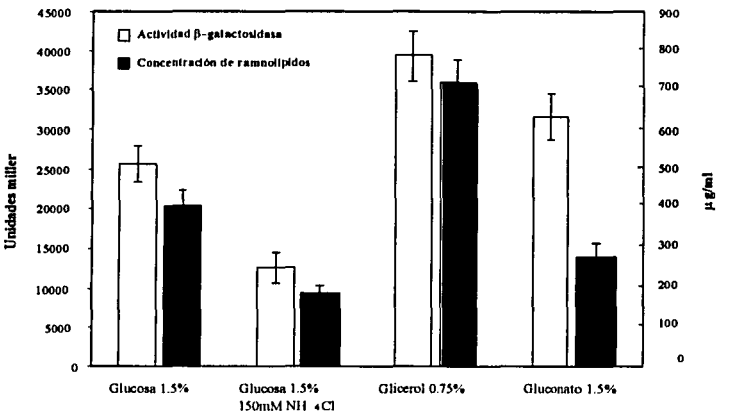

Figura 8. Expresión de *rhlR* y producción de ramnolípidos en fase estacionaria tardía de la cepa PAO1 de *P. aeruginosa* en medio mínimo M9 modificado.

El medio de cultivo PPGAS es un medio limitado en fosfatos utilizado para la producción de ramnolípidos por *P. aeruginosa* (Zhang y Miller, 1992), el cual se utilizó para observar el efecto de la adición de fosfatos y de amortiguador de pH. En dichos experimentos, se encontró que al añadir fosfatos desciende claramente la producción de ramnolípidos, aunque la expresión de *rhlR* solo descienda 33% (Figura 9), lo que sugiere que aunque RhlR es necesario para la producción de ramnolípidos no es suficiente, sino que existe otro factor necesario para la síntesis de ramnolípidos, el cual no está presente cuando *P. aeruginosa* crece en medio rico en fosfatos. El medio PPGAS reportado contiene Tris-HCL como amortiguador de pH, pero se modificó agregando MOPS ó ningún amortiguador, observando que tanto la expresión de *rhlR* como la producción de ramnolípidos aumentan considerablemente al utilizar amortiguador MOPS.

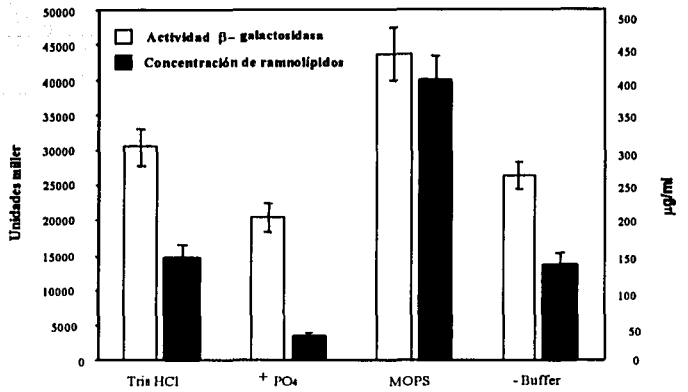

Figura 9. Expresión de *rhlR* y producción de ramnolípidos en fase estacionaria de la cepa PAO1 en medio PPGAS modificado.

Al utilizar el medio rico LB (Figura 10), se observó un fenómeno similar al encontrado en PPGAS/MOPS, sin embargo la producción de ramnolípidos en general en este medio es pobre. Una posible explicación del fenómeno, es que el pH pudiera estar jugando un papel en la estabilización de las proteínas necesarias para la síntesis de ramnolípidos, y además como ha sido reportado (Diggle et. al., 2002), el anillo lactonico de los autoinductores se hidroliza a pH básico, lo que puede estar provocando la baja producción de ramnolípidos en medio LB (donde se alcaliniza el medio) aun cuando *rhlR* se transcriba tanto como en PPGAS.

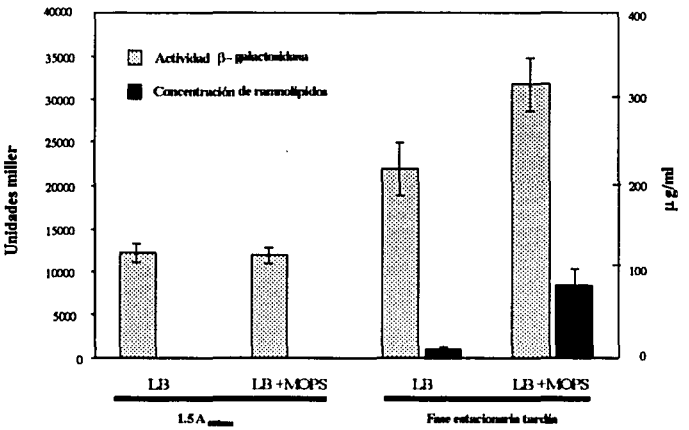

Figura 10. Expresión de *rhlR* y producción de ramnolípidos de la cepa PAO1 en medio LB modificado.

Los resultados sugieren que *rhlR* es un blanco de regulación para algunos factores nutricionales, sin embargo algunos componentes del medio de cultivo, como fosfatos o pH, modulan la producción de ramnolípidos pero no significativamente la expresión de *rhlR*. En el caso del crecimiento en medio LB detectamos un nivel relativamente alto de expresión de *rhlR*, sin embargo, la producción de ramnolípidos fue pobre aun en presencia de buffer MOPS lo que sugiere que en dicho medio la expresión de las enzimas que participan en la producción de ramnolípidos no es limitada por la expresión de *rhlR* sino que existe otro punto de regulación como podría ser la respuesta astringente o el factor sigma alternativo RpoS que se ha observado regulan positivamente algunos genes de la respuesta detectora de quórum (Suh et. al., 1999; van Delden et. al., 2001). Por otro lado, pudiera expresarse un regulador negativo en medio LB que evite la transcripción de *rhlA* hasta fase estacionaria tardía, como MvaT, que recientemente se encontró como un regulador negativo de *lecA*, un gen que codifica para una lectina y está bajo el control del sistema detector de quórum *rhl* (Diggle et. al., 2002).

Otro punto de regulación importante es la producción de autoinductores, ya que su concentración puede ser limitante para la producción de ramnolípidos, en tanto RhlR puede incluso reprimir la producción de ramnolípidos cuando no está acomplejado con BHL (Anexo II).

VII. Conclusiones

I. El operón *rhlAB* se transcribe principalmente en fase estacionaria aún en presencia de RhlR y BHL desde la fase exponencial de crecimiento en medios ricos. Este silenciamiento puede estar relacionado con la ausencia de un activador o con la presencia de algún represor en fase exponencial.

II. El factor determinante de la expresión del promotor *rhlAB* en fase exponencial responde a la limitación de nutrientes.

III. La expresión del promotor del gen *rhlA* es parcialmente dependiente del factor σ^S en tanto σ^{54} no tiene un efecto directo en este proceso como había sido propuesto anteriormente.

IV. La región reguladora de *rhlR* posee cuatro promotores, tres de los cuales responden positivamente a reguladores transcripcionales tales como LasR, Vfr, y RpoN que actuan como reguladores positivos, en tanto *rhlR* posee una autorregulación negativa por RhlR.

V. La región promotora de *rhlR* presenta dos probables cajas *las* y una probable caja Vfr.

VI. Existe una interacción directa y específica entre el regulador transcripcional RhlR y la región promotora de *rhlA*, la cual ocurre tanto con la proteína RhlR sola, como con el complejo RhlR/BHL.

VII. La conformación adoptada por RhlR varía dependiendo de si está unido o no al autoinductor, como se evidenció por las diferencias en la cobertura del promotor de *rhlA* en estas dos condiciones.

VIII. El complejo RhlR/BHL funciona como un regulador positivo de *rhlA*, en tanto RhlR funciona como un regulador negativo.

IX. La expresión de *rhlR* no solo depende de una alta densidad celular, sino de la composición del medio. Esta dependencia correlaciona con la existencia de una compleja estructura de su región promotora.

X. La regulación de la expresión de *rhlR* muestra que es un punto importante en la respuesta del sistema detector de quórum a factores ambientales así como para la producción de ramnolípidos, sin embargo la presencia de RhlR no es suficiente para producirlos.

VIII. Perspectivas

Las perspectivas planteadas en cada una de las partes estudiadas en esta tesis son:

Silenciamiento en fase exponencial del promotor *rhlAB*

1. Determinar cual es el factor responsable del "silenciamiento" en fase exponencial de la expresión de *rhlAB*.

Regulación transcripcional del gen *rhlR*

2. Demostrar que la caja VFR putativa encontrada en la región promotora de *rhlR* es en efecto una secuencia de pegado de esta proteína.

3. Determinar cual o cuales de las dos cajas *las* encontradas en la región promotora de *rhlR* son funcionales, así como determinar si son sitios de pegado de LasR, de RhlR o de ambos.

Mecanismo de regulación transcripcional del promotor *rhlAB* por RhlR

4. Determinar cuales son las bases importantes de la caja *las* de *rhlAB* para la especificidad y afinidad por la proteína RhlR.

Influencia del medio de cultivo en la expresión de *rhlR* y en la producción de ramnolípidos.

5. Medir la concentración de autoinductores en los sobrenadantes de los medios utilizados, para determinar si la síntesis de autoinductores son el factor limitante para la producción de ramnolípidos en algunas condiciones.

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