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Mecanismos moleculares que regulan la expresión de *ompS1* en *Salmonella enterica* serovar Typhi: dos activadores, dos represores y un anti-represor

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A

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RESUMEN

El gen *ompS1* en *S*. Typhi codifica para una porina quiescente, homóloga a las porinas mayoritarias OmpC/OmpF. Los bajos niveles de expresión de esta porina se encuentran mediados por la proteína nucleoide H-NS, que se une directamente a su región reguladora. Además de H-NS, se encontró que StpA, otra proteína nucleoide y parálogo de H-NS, silenció directamente la expresión de *ompS1* en un fondo STY*hns*99. LeuO, un regulador de la familia LysR, contrarrestó el efecto represor de H-NS y StpA, actuando como un antagonista de estas proteínas nucleoides. El mecanismo anti-represor mediado por LeuO es a través de su competencia por una zona que se sobrepone con un sitio de nucleación de H-NS, desestabilizando el complejo nucleorrepresor y finalmente desplazándolo de la región reguladora, donde el regulador de respuesta OmpR actúa como un activador transcripcional, determinado el uso de los promotores de *ompS1*. Además del papel de proteínas reguladoras que controlan la expresión de *ompS1*, se describió que la curvatura intrínseca del DNA tiene un efecto represor sobre la expresión del gen, lo que favorece la formación de una estructura tipo horquilla represora mediado por H-NS. El papel de OmpR sobre la regulación de *ompS1* había sido descrito como un regulador transcripcional, donde la presencia o ausencia activaba o reprimía los promotores. En este trabajo se encontró que su estado de fosforilación es el que determinaba el uso de los promotores: OmpR-P activó y reprimió a P1 y P2, respectivamente. La fosforilación de OmpR aumentó dramáticamente su afinidad a la región promotora de *ompS1*. CpxR, un regulador de respuesta involucrado en estrés de la envoltura celular, se encontró que regula positivamente la expresión de *ompS1* activando el promotor P2 en ausencia de OmpR-P, uniéndose directamente a la región promotora de *ompS1*. CpxR funcionaría como un soporte molecular que actúa en ausencia de OmpR-P, manteniendo la expresión de OmpS1 en la bacteria. Estos datos indican un mecanismo complejo y fino en donde, bajo condiciones represoras, H-NS y StpA (dos represores) fuertemente silencian la expresión de *ompS1*; mientras que bajo condiciones de inducción (probablemente en el huésped) LeuO (un anti-represor) antagoniza este efecto represor, desplazando a H-NS y a StpA de la región reguladora, permitiendo que OmpR o CpxR (dos activadores) regulen positivamente la expresión de *ompS1* controlando la actividad de los promotores.

ABSTRACT

The *S*. Typhi *ompS1* gene encodes a quiescent porin which is a homologue of the OmpC/OmpF majority porins. The low expression levels of *ompS1* are H-NS-mediated, a nucleoid protein that directly binds to the regulatory region. In addition to H-NS, we found that StpA, another nucleoid protein and an H-NS paralogue, directly silenced *ompS1* expression in an STY*hns*99 background. LeuO, a LysR-family regulator, counteracted the repressor effect of H-NS and StpA, acting as an antagonist of these nucleoid proteins. The LeuO-mediated anti-repressor mechanism was by competing for binding in the regulatory region, in a site that overlaps with an H-NS nucleation site, thus destabilizing the nucleorepressor complex and finally displacing H-NS. The OmpR response regulator then acts as a transcriptional activator determining the use of both *ompS1* promoters. In addition to the role of regulatory proteins that control *ompS1* expression, we described that the DNA intrinsic curvature has a repressive effect, favoring the formation of an H-NS mediated loop-type repressor structure. The role of OmpR on *ompS1* regulation had been described as a transcriptional regulator, where its presence or the absence activated or repressed the promoters. In this work we found that the phosphorylation status of OmpR determines the use of both promoters: OmpR-P activates and represses P1 and P2, respectively. OmpR phosphorylation dramatically increased its affinity to the *ompS1* promoter region. CpxR, a response regulator involved in cellular envelope stress, was found to positively regulate *ompS1* expression, activating the P2 promoter in the absence of OmpR-P by directly binding to the *ompS1* promoter region. CpxR would function as a molecular back-up, acting in the absence of OmpR-P thus allowing OmpS1 expression. These data indicate a complex and fine mechanism where, under repressive conditions, H-NS and StpA (two repressors) silence tightly *ompS1*expression; whereas under induction conditions (probably in the host) LeuO (an anti-repressor) antagonizes this repressor effect, displacing both H-NS and StpA from the regulatory region, allowing either OmpR or CpxR (two activators) to positively regulate *ompS1* expression controlling promoter activity.

1. INTRODUCCIÓN

1.1 *Salmonella*

Salmonella es una bacteria Gram-negativa perteneciente a la clase γ de las proteobacterias y forma parte de la familia Enterobacteriaceae. El género *Salmonella* está conformado por dos especies: *bongori* y *enterica* (Penner *et al*., 1988; Brenner *et al*., 2000). *Salmonella enterica* se ha dividido en seis subespecies: *enterica, salamae, arizonae, diarizonae, houtenae e indica* (Christensen *et al*., 1998).

La subespecie más estudiada ha sido *Salmonella enterica enterica* de la cual se han identificado más de 2400 serovariedades que pueden infectar aves, reptiles y mamíferos, incluyendo al humano. Estas serovariedades se han clasificado de acuerdo al esquema básico de Kauffman-White, basado en los antígenos capsulares (Vi), flagelares (H) y el lipopolisacárido (O) (Scherer and Miller, 2001).

Dentro de las serovariedades más importantes, destaca *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), la cual provoca gastroenteritis, diarrea localizada, dolor abdominal y en algunas ocasiones fiebre (Scherer and Miller 2001). *S*. Typhimurium produce una enfermedad similar a la fiebre tifoidea en ratones Balb/c, por lo que se ha usado como un modelo de patogénesis (Zhang *et al*., 2003; Coburn *et al*., 2005; Bowe *et al*., 1998). Otra serovariedad de importancia médica es *Salmonella enterica* serovar Typhi (*S.* Typhi), que es el agente etiológico de la fiebre tifoidea, una enfermedad sistémica y febril, que afecta únicamente al ser humano (Pang *et al*., 1998), y es el modelo bacteriano usado en este trabajo.

1.2 Patogénesis de *Salmonella enterica*

La ruta de infección natural para *Salmonella enterica* es la vía oral, a través de la ingestión de alimentos o aguas contaminadas (Calva *et al*., 1988). En el proceso de infección la bacteria penetra la mucosa del yeyuno y del íleon, a través de las células M. En este proceso se induce la ondulación de la membrana de la célula eucariota lo que facilita la internalización de la bacteria (Fig. 1). Después de un periodo de adaptación (3-4 horas) *Salmonella enterica* puede replicarse en las vacuolas de las células fagocíticas, células que se caracterizan por tener concentraciones limitadas de Mg^{2+}, Fe^{2+} y un pH ácido (Fig. 1). *Salmonella enterica* sobrevive a estas condiciones e induce la apoptosis del macrófago. Posteriormente la bacteria pasa al torrente sanguíneo diseminándose al hígado, bazo, médula ósea, vesícula biliar y placas de

Peyer. La concentración de bacterias en el bazo e hígado aumenta durante 3 o 4 días, provocando bacteremia, choque endotóxico y finalmente la muerte del huésped (Beuzon and Holden, 2001; House *et al*., 2001; Scherer and Miller, 2001; Zhang *et al*., 2003).

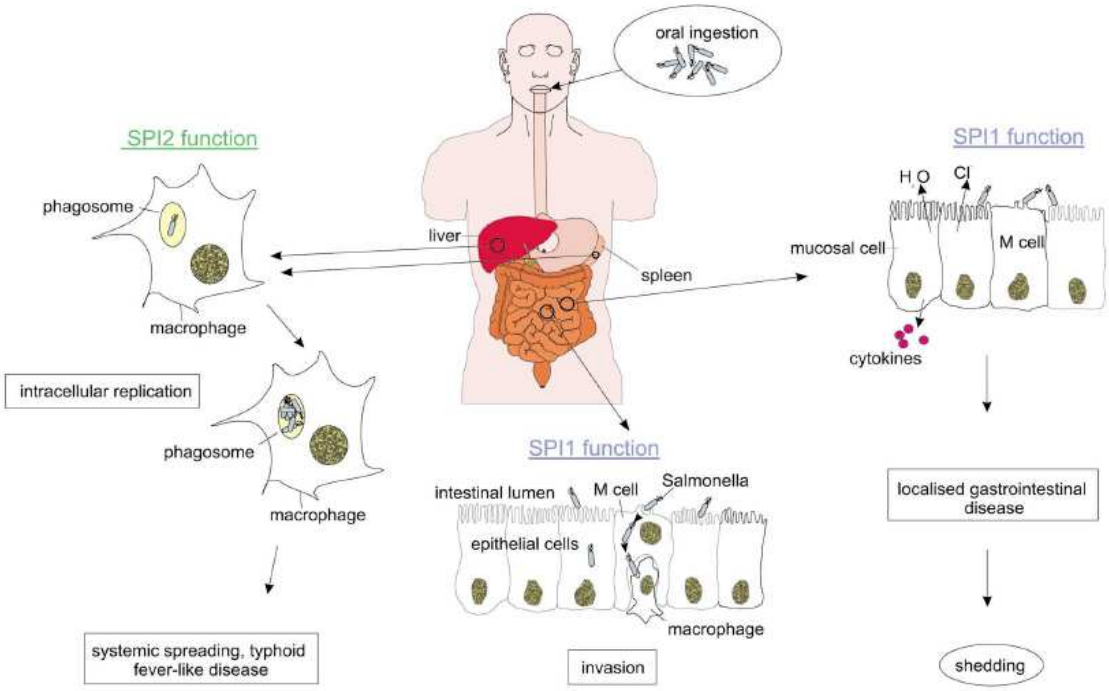

Fig. 1. Representación esquemática de las interacciones patógeno-hospedero durante la patogénesis de *Salmonella enterica*. La función de SPI-1 es requerida para las etapas tempranas de la infección, como por ejemplo la entrada a células no fagocíticas, como son células epiteliales. SPI-1 es requerida para la formación de la diarrea localizada. La función de SPI-2 es requerida para las etapas tardías de la infección, por ejemplo, la diseminación y colonización de órganos del hospedero. Imagen tomada de Hansen-Wester and Hensel, 2001.

Para que lleve a cabo todos los procesos de patogénesis en el hospedero, *Salmonella enterica* contiene en su genoma, genes que se requieren para su virulencia. En este contexto, el genoma de *S*. Typhimurium está compuesto de 4,867 genes y se ha calculado que alrededor del 4% de su genoma se encuentra involucrado en la virulencia (Bowe *et al*., 1998). Los genes localizados en las islas de patogenicidad contribuyen en la patogénesis de *Salmonella enterica.* Estas islas están constituidas por un grupo de genes que codifican factores específicos de virulencia, donde su porcentaje de G+C es diferente del resto del genoma y frecuentemente se encuentran insertadas cerca de RNA de transferencia o secuencias de inserción (Ochman and Groisman, 1996; Marcus *et al*., 2000). *Salmonella enterica* tiene varias islas de patogenicidad (SPI), dentro de las cuales SPI-1 y SPI-2 son las más importantes y estudiadas. Estas islas codifican cada una para un sistema de secreción tipo III, que son agujas moleculares que inyectan diversas moléculas efectoras del citoplasma bacteriano al citosol eucariótico, alterando diversas vías de señalización de la célula huésped (Fig. 1). SPI-1 está implicada en la invasión celular, principalmente

en células no fagocíticas como son las células epiteliales, produciendo el llamado “ruffling” u ondulación en la membrana (Fig. 1; Zhou and Galán, 2001). Se ha reportado que los genes de SPI-2 están involucrados en la infección sistémica y en la sobrevivencia dentro del macrófago (Fig. 1; Hansen-Wester and Hensel, 2003; Waterman and Holden, 2003). La información codificada en las islas de patogenicidad es relevante en la infección y en la sobrevivencia del patógeno en el hospedante. Además de las islas de patogenicidad, existen otros genes que se requieren y participan en la virulencia de *Salmonella enterica*, como es el caso de genes que codifican para proteínas de la membrana externa (interacción con células eucarióticas), así como reguladores transcripcionales globales.

1.3 Porinas: porteros de la célula

La membrana externa es una característica distintiva de las bacterias Gram-negativas. Las porinas son proteínas de la membrana externa (PME) que producen poros formando diferentes tipos de canales: sustratos-específicos (LamB), ion-selectivos (PhoE) o no específicos (OmpF). Estos poros permiten el transporte de pequeñas moléculas hidrofílicas (~600 Da) y la expulsión de productos de desecho. Las porinas también expulsan muchos antibióticos e inhibidores como sales biliares (Fig. 2; Nikaido, 2003).

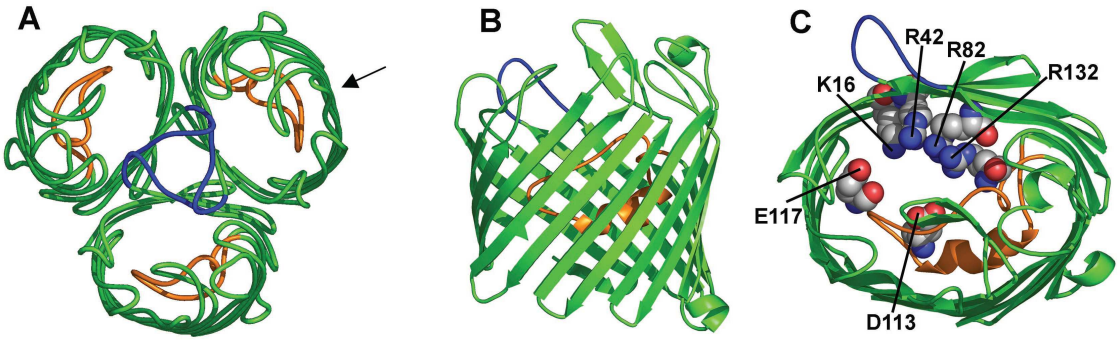

Fig. 2. Estructura de la porina OmpF de *E. coli*. A) Vista superior del trímero en dirección perpendicular al plano de la membrana. B) Vista lateral de la unidad monomérica aproximadamente en dirección de la flecha del panel A. C) Vista superior de la unidad monomérica, mostrando la abertura de la región estrecha del canal y los aminoácidos relevantes para su formación. Imagen tomada de Nikaido, 2003.

Las porinas de la familia de Porinas Bacterianas Generales (PBG, Número de Clasificación de Transportadores 1.B.1), incluyen a OmpC y OmpF de *Escherichia coli* (Saier *et al*., 2006; 2009) que forman trímeros estables con ligera preferencia hacia cationes. En *E. coli* OmpF parece tener un poro más grande que OmpC (Hancock *et al*., 1987). OmpC y OmpF son las porinas mayoritarias. La porina PhoE tiene una

preferencia general para aniones pero no es fosfato-específica, aunque su producción se induce bajo limitación de fosfato (Nikaido, 2003). *Salmonella enterica* tiene, además de OmpC y OmpF, dos porinas quiescentes de la familia PBG, nombradas OmpS1 y OmpS2. Los homólogos de los genes *ompS1* y *ompS2* en *Escherichia coli* son los genes *yedS* y *ompN*, respectivamente (Fernández-Mora *et al*., 1995; 2004). En este respecto, en *E. coli* y otras enterobacterias, OmpG es una porina quiescente involucrada en el importe y metabolismo de oligosacáridos (Fajardo *et al*., 1998). *S*. Typhimurium posee una porina abundante característica, OmpD, otro miembro de la familia PBG. En adición, hay otras pequeñas porinas monoméricas como OmpA, OmpX y OmpW (Nikaido, 2003; ver TCDB www.tcdb.org).

En términos de virulencia, la ausencia de ciertas porinas disminuye la patogénesis tanto en las etapas tempranas (interacción con enterocitos) como en las tardías de la infección (sobrevivencia intracelular). Los fenotipos asociados a la virulencia de algunas porinas son resumidos en la Tabla 1.

Tabla 1. Fenotipos asociados a la virulencia de genes de porinas

Porina	Bacteria	Fenotipo en virulencia	Referencias
OmpC	*S*. Typhimurium	Una mutante en el gen *ompC* está disminuida en la invasión a macrófagos múridos. Una doble mutante *ompC ompF* está atenuada en la virulencia hacia ratones Balb/c.	Negm and Pistole, 1999; Chatfield *et al*., 1991
	Shigella flexneri	OmpC está involucrado en la sobrevivencia intracelular.	Bernardini *et al*., 1993
OmpF	*S*. Typhimurium	Una doble mutante *ompC ompF* está atenuada en la virulencia hacia ratones Balb/c.	Chatfield *et al*., 1991
OmpD	*S*. Typhimurium	Una mutante en el gen *ompD* está afectada en la invasión a macrófagos humanos y células epiteliales.	Hara-Kaonga and Pistole, 2004
OmpX	*Yersinia pestis*	Una mutante *ompX* muestra disminución tanto en la adherencia como en la internalización a monocapas de células Hep-2 y es más susceptible al efecto bactericida del suero humano. Además, la ausencia de OmpX reduce significativamente el fenotipo de autoagregación y pérdida de formación de películos *in vitro*	Kolodjziejek *et al*., 2007
OmpS1	*S*. Typhimurium	Una mutante *ompS1* está atenuada en la virulencia hacia ratones Balb/c por vía oral.	Rodríguez-Morales *et al*., 2006
OmpS2	*S*. Typhimurium	Una mutante *ompS2* está atenuada en la virulencia hacia ratones Balb/c por vía oral.	Rodríguez-Morales *et al*., 2006

Con respecto a la respuesta a estrés, las mutantes deficientes en OmpR, el regulador maestro de las porinas, crecen más lento que la cepa silvestre a baja osmolaridad y a pH ácido. De manera similar, mutantes en los genes *ompC* y *ompF* son también deficientes en el crecimiento a alta osmolaridad y a pH alcalino (Sato *et al*., 2000; Kaeriyama *et al*., 2006). Las porinas OmpD y OmpW en *S*. Typhimurium se

han involucrado en la destoxificación celular, por medio de la expulsión de compuestos tóxicos tales como el metil viológeno que causan estrés oxidativo (Santiviago *et al*., 2002; Gil *et al*., 2007).

1.4 OmpR, regulador maestro de porinas

OmpR es el regulador de respuesta citoplásmico de un sistema de dos componentes, que incluye a EnvZ, una histidín cinasa alojada en la membrana interna. OmpR es un regulador pleiotrópico que controla la expresión de genes de porinas y de procesos celulares como la quimiotáxis y la virulencia (Slauch and Silhavy 1989; Chatfield *et al*., 1991; Shin and Park 1995; Lee *et al*., 2000; Garmendia *et al*., 2003; Park and Forst 2006; Brzosteck *et al*., 2007). La proteína OmpR contiene dos dominios: el amino terminal contiene el sitio de fosforilación (D55) y el carboxilo terminal la función de unión a DNA en un motivo hélice-vuelta-hélice tipo aleta (Fig. 3; Kato *et al*., 1989). El estado de fosforilación de OmpR es crucial para la regulación de sus genes blanco. EnvZ es la principal cinasa que fosforila OmpR. Las señales ambientales descritas que influyen en la actividad de EnvZ son la osmolaridad, el pH, la temperatura, los nutrientes y las toxinas (Forst and Inouye, 1988; Bang *et al*., 2000; Liu and Ferenci 2001). Sin embargo, bajos ciertas condiciones, el acetil fosfato puede funcionar como donador de fosfato para OmpR (Russo and Silhavy 1991; Hsing and Silhavy 1997; Bang *et al*., 2000). Además de su actividad cinasa, EnvZ también actúa como fosfatasa, controlando la concentración de OmpR-P en la célula (Qin *et al*., 2001). OmpR-P es la forma activa de OmpR, donde la fosforilación incrementa su afinidad de unión a DNA para las regiones promotoras de los genes *ompC* y *ompF* (Forst *et al*., 1989; Huang *et al*., 1997; Head *et al*., 1998). Además de su papel activador, OmpR-P actúa también como represor transcripcional de ciertos genes (Shin and Park, 1995; Brzosteck *et al*., 2007). OmpR actúa como dímero, vía oligomerización de hojas β y la unión a DNA en una orientación cabeza a cabeza (Fig. 3; Maris *et al*., 2005). El sitio de unión consenso para OmpR consiste de 20 pares de bases (pb), esto es, para un dímero de OmpR-P (Harlocker *et a*l., 1995; Harrison-McMonagle *et al*., 1999; Yoshida *et al*., 2006).

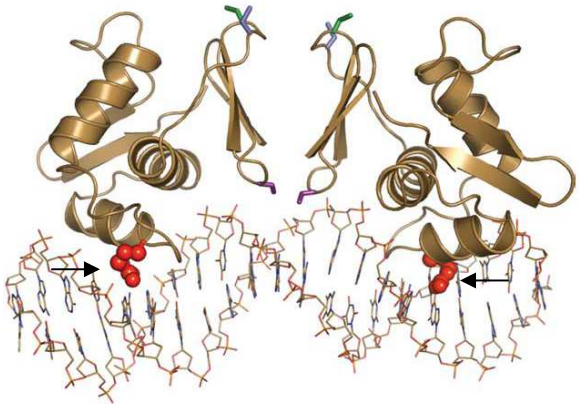

Fig. 3. Modelo del complejo OmpR-DNA. El modelo cabeza a cabeza de la dimerización de OmpR hacia el DNA es mostrado. La arginina 190 que es crucial para la interacción con el surco mayor del DNA está indicada con una flecha. Imagen tomada de Maris *et al*., 2005.

En *E.coli* K-12 a baja osmolaridad, la concentración de OmpR-P es baja, pero la suficiente para interactuar con sitios de alta afinidad (F1-F2-F3) en la región reguladora de *ompF* que son responsables para la activación de su transcripción. En alta osmolaridad, la concentración de OmpR-P es mucho mayor y permite que OmpR-P ocupe los sitios en la región reguladora de *ompC* responsables de su activación transcripcional, y al sitio en *ompF* (F4) responsable para su represión transcripcional (Pratt *et al*., 1996).

Este modelo predice que la regulación de *ompC* y *ompF* es una consecuencia directa de los niveles de OmpR-P en la célula y es dependiente sobre el modo en que OmpR-P interactúa con los sitios en las regiones regulatorias de *ompC* y *ompF*. Un trabajo reciente describió un nuevo modo de unión de OmpR-P a las regiones regulatorias de *ompC* y *ompF* llamado "modelo galopeante" (Fig. 4). Este mecanismo implica una fina unión jerárquica de OmpR, permitiendo una regulación diferencial de la transcripción de *ompC* y *ompF*, que minimiza su expresión conjunta la cual responde a cambios en la osmolaridad del medio (Yoshida *et al*., 2006).

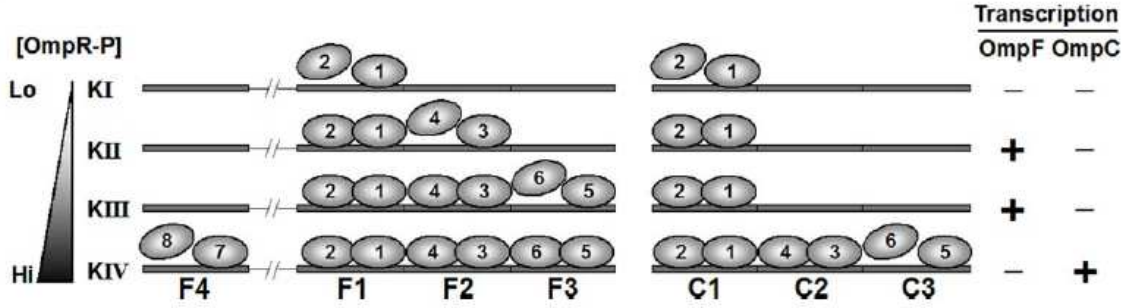

Fig. 4. Modelo galopeante regulatorio de OmpR-P sobre los genes de porinas. El modelo ilustra la unión cooperativa de OmpR-P a las regiones regulatorias de *ompC* y *ompF*. La forma oval enumerada representa una molécula de OmpR-P. La ocupación de OmpR-P sobre los sitios C1-C2-C3 son necesarios para la activación transcripcional de *ompC*.Los sitios F1-F2-F3 son necesarios para la activación transcripcional para *ompF*, donde el sitios F4 es crucial para su represión. Imagen tomada de Yoshida *et al*., 2006.

Este modelo de expresión recíproca no aplica a *S*. Typhi y a *Shigella flexneri*, donde la producción de OmpC es constitutiva (Puente *et al*., 1991; Bernardini *et al*., 1993). La expresión constitutiva de *ompC* tanto de *S*. Typhi y *E. coli* en un fondo genético *S*. Typhi es dependiente de la presencia de EnvZ de *S*. Typhi. (Martínez-Flores *et al*., 1999). La inducción por alta osmolaridad se ha observado para *ompX* en *Enterobacter aerogenes*, donde dos cajas de unión se han identificado *in silico* (Dupont *et al*., 2004).

Además de regular la síntesis de porinas en *Salmonella*, OmpR activa la expresión de SPI-2 a través del sistema de dos componentes SsrAB (Lee *et al*., 2000), por lo que una mutante *ompR* en *S*. Typhimurium está atenuada en la virulencia en el modelo del ratón (Chatfield *et al*., 1991).

1.5 El sistema Cpx como respuesta al estrés en la envoltura celular

El sistema Cpx forma un sistema de dos componentes, donde CpxA es una histidín cinasa transmembranal y CpxR es el regulador de respuesta (Danese *et al*., 1995; Raivio and Silhavy 1997). CpxA fosforila a CpxR, donde CpxR-P activa o reprime transcripcionalmente un grupo de genes involucrados en la respuesta de la bacteria hacia los estrés causados en la envoltura celular como el pH alcalino, la sobreexpresión de proteínas de membrana y las alteraciones en el ambiente que perturben la membrana celular. El sistema Cpx también se ha involucrado en regular la formación de biopelículas, controlar factores de virulencia y mediar la adherencia de la bacteria a células eucarióticas a través del ensamblaje de estructuras sobre la superficie celular como pilis, adhesinas o sistemas de secreción (Fig. 5; Raivio 2005).

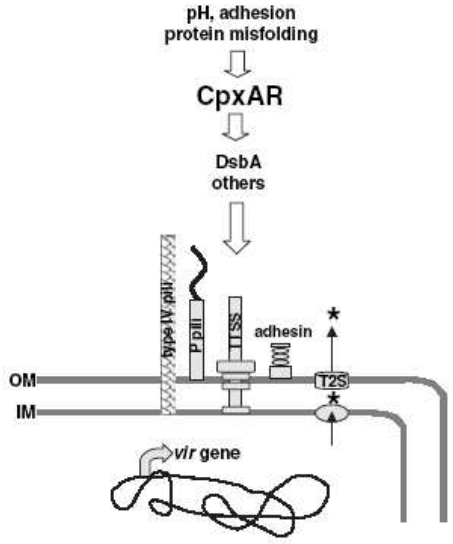

Fig. 5. El sistema Cpx como respuesta de la bacteria hacia el estrés en la envoltura celular. La respuesta mediada por CpxAR induce la expresión de una variedad de genes, incluyendo DsbA y otras proteínas que catalizan el plegamiento y degradación de proteínas de envoltura. La inducción de CpxAR tiene efectos sobre el ensamblaje y expresión de los pilis P y tipo IV, así como también en la regulación genética en numerosos patógenos. DsbA afecta los pilis, SST3 (sistema de secreción tipo III), SST2 (sistema de secreción tipo II) y adhesinas de membrana externa. Imagen tomada de Raivio, 2005.

Además de todas estas funciones, el sistema Cpx controla la expresión de las porinas mayoritarias OmpC y OmpF. En *E*. *coli*, una mutante *cpxA* incrementó la transcripción de *ompC* y reprimió fuertemente la transcripción de *ompF* bajo condiciones en que los niveles de acetil fosfato son altos. Datos genéticos mostraron que este fenotipo fue dependiente de la fosforilación del regulador de respuesta CpxR, esto es, en ausencia de CpxA y en condiciones de cultivo donde los niveles de acetil fosfato son altos, los niveles de CpxR-P aumentan y se acumulan debido a que CpxA es la principal fosfatasa que desfosforila a CpxR-P. Mediante ensayos de protección a DNasa I se mostró que CpxR-P se unió a secuencias cercanas al promotor sobrelapando con los sitios de unión a OmpR, y también se unió a secuencias lejanas a los promotores (~350 pb) tanto de *ompC* y *ompF* (Batchelor *et al*., 2005).

Además de los genes de porinas clásicos, OmpR y CpxR co-regulan otros genes como: *nanC* que codifica para un canal de membrana externa que transporta

ácido N-acetilneuramínico en *E. coli* (Condemine *et al*., 2005), *csgD* que codifica para el regulador positivo de la síntesis del curli (adhesina) en *E. coli* (Jubelin *et al*., 2005) e *inv* que codifica para la invasina, una proteína de membrana externa que es un factor de virulencia en *Yersinia* (Carlsson *et al*., 2007; Brzosteck *et al*., 2007).

1.6 H-NS, el centinela del genoma

H-NS es una proteína de 137 aminoácidos (~15 kDa) de unión a DNA que es abundante (~20,000 copias por célula) y se asocia al nucleoide bacteriano (Atlung and Ingmer, 1997; Dorman, 2004). H-NS contiene un dominio de oligomerización en el amino terminal y un dominio de unión a DNA en el carboxilo terminal; ambos dominios se encuentran conectados por un enlazador flexible (Dorman 2004; Rimsky 2004). H-NS en solución forma dímeros que tienen la capacidad de crear puentes DNA-H-NS-DNA ya sea entre moléculas de DNA separadas o entre diferentes regiones de la misma molécula de DNA (Dorman 2007; Noom *et al*., 2007). H-NS es un regulador maestro que reprime la transcripción de una porción significante del genoma en enterobacterias (5-12%) (Hommais *et al*., 2001; Navarre *et al*., 2006; Lucchini *et al*., 2006; Baños *et al*., 2008).

La expresión de la proteína H-NS es relativamente constante en *E. coli* y en *Salmonella* (Hinton *et al*., 1992; Free and Dorman 1995). El gen *hns* se autorregula negativamente y también es reprimido por StpA (Hinton *et al*., 1992; Dersch *et al*., 1993; Falconi *et al*., 1993; Free and Dorman, 1995). Fis (Factor for Inversion Stimulation) y la proteína de choque frio CspA (Cold shock protein) activan el promotor de *hns* (La Teana *et al*., 1991). DsrA, un pequeño RNA regula negativamente la expresión del RNA mensajero (mRNA) de *hns* en conjunto con la proteína Hfq, un regulador pleiotrópico que se une a mRNA (Lease and Belfort, 2000).

H-NS debe ser visto como un determinante de la arquitectura cromosomal más que un componente estructural general, ya que análisis de nucleoides de *E. coli* tratados con urea sugieren que ni H-NS ni otras proteínas asociadas al nucleoide como Fis, Dps o StpA se requieren para una transición cooperativa entre formas compactadas o parcialmente expandidas del cromosoma (Zimmerman, 2006). Estudios iniciales sugerían que H-NS tenía solamente un papel estructural en la organización del cromosoma bacteriano, sin embargo, estudios recientes en *S.* Typhimurium han mostrado que H-NS funciona como un guardián del genoma. H-NS se une a regiones del genoma que tienen alto contenido de A+T. Esas regiones incluyen islas de patogenicidad, que codifican para los principales determinantes de virulencia de *Salmonella*, y las cuales se ha postulado que se adquirieron por

transferencia horizontal. La hipótesis es que H-NS actúa como un silenciador de los genes adquiridos por transferencia horizontal, permitiéndolos ser incorporados dentro del genoma en un estado quiescente, evitando su expresión descontrolada que comprometa la viabilidad celular (Fig. 6; Lucchini *et al*. 2006; Navarre *et al*., 2006; 2007; Dorman, 2007). Mutantes nulas *hns* no son viables en *Salmonella*, donde las mutantes truncadas obtenidas (con un carboxilo terminal funcional de H-NS), están atenuadas en la virulencia en el modelo del ratón (Harrison *et al*., 1994). Observaciones similares se han hecho para *Yersinia*, donde no ha sido posible construir mutantes nulas *hns* (Heroven *et al*., 2004; Ellison and Miller, 2006).

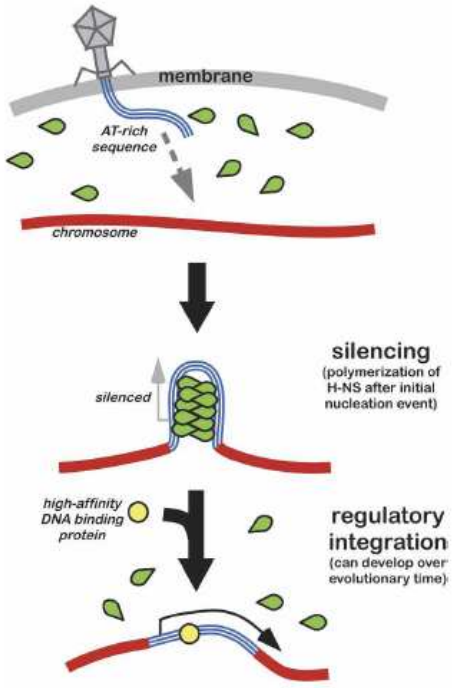

Fig. 6. H-NS silencía el DNA extraño adquirido por transferencia horizontal. La unión de H-NS a DNA extraño rico en A+T silencía su expresión. Una proteína de unión a DNA específica de secuencia con mayor afinidad que H-NS puede competir con éste y contrarrestar su silenciamiento permitiendo la expresión genética bajo condiciones adecuadas. De esta forma, el silenciamiento xenogenético permite que la célula bacteriana huésped pueda tolerar la presencia de secuencias extrañas y básicamente recluta y adapta la secuencia dentro de una red regulatoria pre-existente. Imagen tomada de Navarre *et al*., 2007.

Muchos esfuerzos se han dado para comprender la secuencia nucleotídica que reconoce H-NS sobre el DNA a pesar que se han descrito muchos genes regulados por esta proteína nucleoide. Un estudio reciente ha identificado una secuencia nucleotídica discreta 5-TCGATATATT-3' (Fig. 7) al que H-NS se une con mayor afinidad que a otros elementos ricos en A+T (Lang *et al*., 2007).

Fig. 7. Secuencia consenso para la unión de H-NS. Representación Logo de los motivos de unión de H-NS determinados experimentalmente y de genes expresados diferencialmente en un fondo *hns* bajo diferentes condiciones de crecimiento. Imagen tomada de Lang *et al*., 2007.

Probablemente esa secuencia o secuencias relacionadas, pueden formar sitios de nucleación, que son sitios de unión de alta afinidad para la proteína H-NS, donde puede extenderse lateralmente en todo el DNA, formando filamentos que dan origen a estructuras tipo horquillas o puentes DNA-H-NS-DNA (Fig. 8; Rimsky *et al*., 2001; Lang *et al*., 2007).

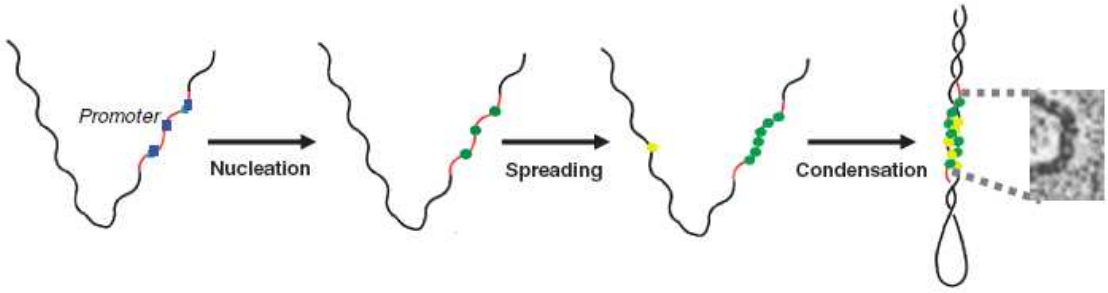

Fig. 8. Mecanismo propuesto para el silenciamiento transcripcional mediado por H-NS. Los sitios de nucleación de alta afinidad de unión para H-NS promueven la extensión (polimerización) y condensación de H-NS sobre DNA superenrrollado para generar una fibra silenciada (formada por DNA vecinos). Imagen tomada de Lang *et al*., 2007.

Además de la secuencia nucleotídica, se ha reportado que la curvatura intrínseca del DNA puede facilitar la formación de horquillas, donde también la curvatura es sensible a condiciones ambientales como la temperatura y la osmolaridad. Para el promotor *virF* en *Shigella flexneri* se demostró que el incremento de la temperatura puede reducir el grado de curvatura y desplazar el centro de la curvatura en formas que disminuyan la capacidad de H-NS para mantener una estructura tipo puente (Prosseda *et al*., 2004).

Respecto a la regulación de porinas, H-NS reprime la expresión de *ompC* directamente y regula positivamente la expresión de *ompF*, ya que en ausencia de H-NS, se desreprime la expresión de *micF* que codifica para el RNA antisentido del mRNA de *ompF* (Suzuki *et al*., 1996).

1.7 StpA como soporte molecular de H-NS

La proteína StpA se identificó originalmente sobre la base de su capacidad para suprimir el fenotipo Td^- de una mutante en el intrón Td (timidilato sintasa) del fago T4 deficiente en el "splicing" (Zang and Belfort, 1992), y consecuentemente aislado por su capacidad para complementar mutantes *hns* modulando la expresión del gen de arginina descarboxilasa (Shi and Bennet, 1994). Análisis bioquímicos mostraron que StpA estimula el "splicing" *in vitro* del intron *td* del grupo T4 actuando como chaperona de RNA que actúa sobre RNA mal estructurado y de esa manera promover la

formación de la estructura catalíticamente activa del intrón autoprocesado (Zhang *et al*., 1995)

En *Escherichia coli* y otras enterobacterias relacionadas, tales como *S.* Typhimurium, se encuentra StpA, un parálogo de H-NS (Shi and Bennett, 1994; Sondén and Uhlin, 1996; Zhang *et al*., 1996). StpA tiene 133 aminoácidos que muestra 58% de identidad y 63% similitud con H-NS. Sin embargo, StpA es más básica que H-NS con un pI predicho de 9.08 comparado con 5.25 para H-NS. StpA se une tanto a DNA como a RNA, y también muestra preferencias por secuencias de DNA intrínsicamente curvadas (Zhang *et al*., 1996; Sonnefield *et al*., 2001). Se ha mostrado que los genes *hns* y *stpA* exhiben autorregulación negativa cruzada, esto es, el producto de cada gen puede inhibir tanto su propio gen como el del otro (Zhang *et al*., 1996). Sin embargo, el efecto más fuerte parece ser la represión de *stpA* por H-NS; esto causa que el nivel de expresión de este gen en una célula sea muy bajo y sugiere que StpA probablemente actúa como un "sustituyente molecular" de H-NS, que es expresado sólo cuando ésta última está ausente (Sondén and Uhlin, 1996; Zhang *et al*., 1996).

Estudios genéticos y bioquímicos han mostrado que H-NS y StpA pueden formar heterodímeros (Williams *et al*., 1996; Johansson and Uhlin 1999; Deighan *et al*., 2000; Dorman 2004). La formación de heterodímeros H-NS-StpA protege a StpA de la degradación proteolítica mediada por Lon, donde una diferencia clave en la secuencia aminoacídica en la posición 21 de la proteína contribuye a la sensibilidad de StpA por Lon (StpA tiene una fenilalanina en esa posición mientras que H-NS tiene una cisteína) (Johansson and Uhlin, 1999).

No hay evidencia que StpA interfiera o amplifique la capacidad de H-NS para reprimir la transcripción, entonces el papel biológico de los heterodímeros H-NS-StpA no es claro. En un estudio en *E. coli* uropatógena (UPEC) usando análisis de microarreglos observaron que una mutante *stpA* no muestra fenotipo, sin embargo, en una doble mutante *hns stpA* se desregulan un grupo de genes diferentes comparados al fondo mutante *hns*, sugiriendo que los fenotipos de StpA pueden ser vistos principalmente en ausencia de H-NS (Müller *et al*., 2006). StpA puede reprimir su propia expresión pero no puede reprimir al operón *bgl*, al menos que el dominio amino terminal de H-NS esté presente para permitir la formación de heterodímeros, es decir, en este caso StpA actúa como adaptador molecular de H-NS (Free *et al*., 2001). StpA junto a H-NS inhiben la formación del complejo abierto de genes que codifican para un sistema de secreción tipo II requeridos para la secreción de una enterotoxina inestable a la temperatura (Yang *et al*., 2007). Además de su efecto represor sobre el inicio de la transcripción, se encontró que StpA regulaba positivamente la expresión del gen de

porina *ompF* por medio de la estabilización de MicF, el RNA antisentido para le mRNA de *ompF* (Deighan *et al*., 2000)

1.8 LeuO, un antagonista de H-NS

El gen *leuO* se localiza entre los operones *ilvIH* y *leuABCD*, involucrados en la biosíntesis de isoleucina-valina, y leucina, respectivamente (Henikoff *et al*., 1988). LeuO es un regulador de la familia LysR de 314 aminoácidos que presenta un dominio de unión a DNA en el amino terminal y un dominio de dimerización/multimerización hacia el carboxilo terminal.

LeuO juega un papel durante la patogénesis, puesto que mutantes en el gen *leuO* causan atenuación para la virulencia de *S*. Typhimurium hacia ratones Balb/c (Rodríguez-Morales et al., 2006; Lawley *et al*., 2006; Chaudhuri *et al*., 2009). De manera similar, este efecto fue visto usando como modelo de infección a *Caenorhabditis elegans* (Tenor *et al*., 2004). En *Vibrio cholerae* una mutante *leuO* disminuye la capacidad de formar biopelículas (Moorthy and Watnick, 2005).

Las condiciones reportadas que inducen la expresión del gen *leuO* son: la fase estacionaria de crecimiento, la limitación de nutrientes y la presencia de la alarmona ppGpp (Fang and Wu, 1998; Fang *et al*., 2000; Majumder *et al*., 2001). Estas condiciones han implicado a LeuO en la respuesta bacteriana al estrés donde probablemente sea importante para la sobrevivencia en ambientes naturales fuera del laboratorio.

El modelo transcripcional de la regulación mediada por LeuO se ha estudiado principalmente en su autorregulación positiva (Fig. 9). Para ello se utilizó un promotor mutado en el gen *leuABCD* (p*leuO-500*), que es divergente a *leuO* confiriéndole auxotrofía hacia leucina. Bajo este sistema se propone que el locus *leuO* es parte de un mecanismo de relevo del promotor, donde Lrp (Leucine-responsive protein), una proteína involucrada en respuesta al ayuno, activa el promotor *ilvIH* provocando un cambio en el superenrollamiento local del DNA que amplifica la activación del promotor de *leuO*. H-NS silencia la expresión de *leuO* por medio de la formación de un núcleofilamento represor. Esta estructura nucleoproteínica es estabilizada por la región AT8, que es un sitio de alta afinidad para H-NS. LeuO se une a dos regiones con alta afinidad (AT7 ubicada entre el promotor y la región AT8, y AT3 localizada entre AT8 y p*leu-500*). Un tercer sitio de unión a LeuO con baja afinidad se detectó en secuencias abajo del gen estructural de *leuO*. LeuO actúa delimitando la represión transcripcional mediada por H-NS formando un puente uniéndose a AT7 y a la región secuencia

debajo de la región codificante de *leuO*, evitando el progreso del nucleofilamento represor hacia el promotor de *leuO* (Fig. 9; Chen *et al*., 2001; 2003; Chen and Wu, 2005).

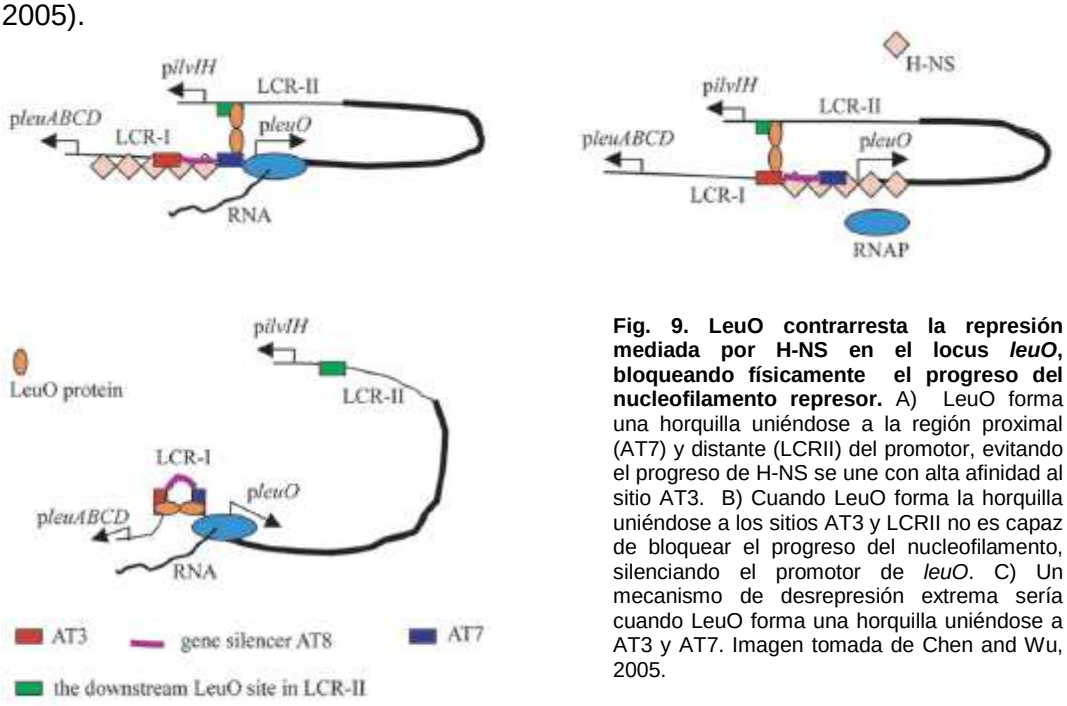

Fig. 9. LeuO contrarresta la represión mediada por H-NS en el locus *leuO*, bloqueando físicamente el progreso del nucleofilamento represor. A) LeuO forma una horquilla uniéndose a la región proximal (AT7) y distante (LCRII) del promotor, evitando el progreso de H-NS se une con alta afinidad al sitio AT3. B) Cuando LeuO forma la horquilla uniéndose a los sitios AT3 y LCRII no es capaz de bloquear el progreso del nucleofilamento, silenciando el promotor de *leuO*. C) Un mecanismo de desrepresión extrema sería cuando LeuO forma una horquilla uniéndose a AT3 y AT7. Imagen tomada de Chen and Wu, 2005.

La sobreexpresión de LeuO afecta la expresión de varios genes, donde la mayoría son regulados por H-NS. LeuO contrarresta el silenciamiento del operón *bgl*, operón involucrado en la utilización de ciertos β-glucósidos, donde H-NS lo reprime (Ueguchi *et al*., 1998); suprime el efecto de una mutación en *hns* en *cadC*, que codifica para el activador de la lisina descarboxilasa inducible por ácido (Shi and Bennet, 1995). LeuO también tiene un efecto negativo sobre la expresión de DsrA, un RNA regulatorio pequeño involucrado en la traducción de RpoS (Klauck *et al*., 1997).

En nuestro laboratorio, se identificó a LeuO como un regulador positivo de la expresión de *ompS2*, un gen que codifica para otra porina quiescente. Para *ompS2*, LeuO junto a OmpR son necesarios para activar su expresión en *S*. Typhi (Fernández-Mora *et al*., 2004). En *Yersinia enterocolitica* se encontró que LeuO activaba la expresión de *rovA*, un gen que codifica a el regulador central de virulencia en esta bacteria (Lawrenz and Miller, 2007). Para *ompS2* se ha visto que en *E. coli* y *S*. Typhimurium, H-NS reprime su expresión (Hommais *et al*., 2001; Navarre *et al*., 2006); para el caso de *rovA* en *Yersinia*, H-NS igualmente reprime la expresión de este gen de virulencia (Heroven *et al*., 2004). Debido a que LeuO activa genes que son reprimidos por H-NS, se ha propuesto que LeuO actúa como un antagonista de H-NS contrarrestando su efecto represor.

2. ANTECEDENTES

2.1 El gen *ompS1*

El gen *ompS1* codifica para una porina que se encuentra alojada en la membrana externa. La región codificante de *ompS1* comprende 1185 pb (394 aminoácidos), tiene un péptido líder de 21 aminoácidos que se procesa dando una forma madura de 373 aminoácidos (41kDa) (Fernández-Mora *et al*., 1995). En el contexto genético, hacia el 5' se encuentra divergente un gen que codifica para una proteína de membrana putativa y hacia el 3' un gen que codifica para proteína de choque frío. Este gen se aisló en el laboratorio usando el gen *ompF* de *E. coli* como prueba heteróloga en un ensayo tipo Southern blot hibridando el genoma de *S*. Typhi (Fernández-Mora *et al*., 1995). El homólogo de *ompS1* (*yedS*) se encuentra truncado en *E. coli* (tiene un codón de termino UAG en la posición L162) (Oropeza *et al*., 1999). Por medio de preparaciones de proteínas de membrana externa se encontró que la porina OmpS1 no se detectó en un gel teñido con Coomassie, a comparación de las porinas mayoritarias OmpC y OmpF. Cuando el gen *ompS1* se clonó a partir de un promotor inducible por IPTG, la porina OmpS1 se detectó abundantemente en la membrana externa, indicando que en las condiciones del laboratorio usadas, OmpS1 es una porina quiescente (Fernández-Mora *et al*., 1995).

2.2 OmpR activa la expresión de *ompS1*

El análisis de remociones hacia el 5' de la región reguladora de *ompS1* fusionadas al gen reportero *lacZ*, mostró que la remoción gradual de secuencias de -310 a -88 corriente arriba del sitio de inicio de la transcripción P1, resultó en un incremento en la expresión de *ompS1*, es decir, las secuencias entre -310 y -88 contenían elementos en *cis* involucrados en el control negativo del gen. La eliminación de secuencias corriente debajo de -88 disminuyó la expresión de *ompS1* indicando que esos nucleótidos son relevantes para su regulación positiva (Fig. 10). La ausencia de OmpR disminuyó significativamente la expresión de las fusiones recortadas hacia el 5', indicando que OmpR, en adición a las porinas mayoritarias OmpC y OmpF, regulaba positivamente la expresión de *ompS1* (Oropeza *et al*., 1999).

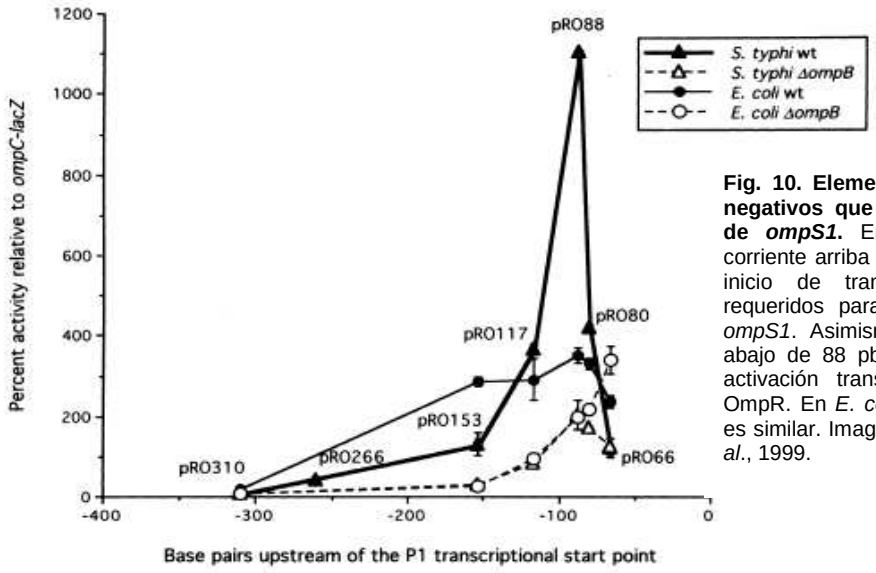

Fig. 10. Elementos en *cis* positivos y negativos que controlan la expresión de *ompS1*. En *S*. Typhi, secuencias corriente arriba de 88 pb con respecto al inicio de transcripción de P1 son requeridos para el control negativo de *ompS1*. Asimismo, secuencias corriente abajo de 88 pb son necesarias para la activación transcripcional mediada por OmpR. En *E. coli* el perfil transcripcional es similar. Imagen tomada de Oropeza *et al*., 1999.

Se determinó que la expresión de *ompS1* fue dependiente de dos promotores sobrepuestos: P1 y P2. El primero de ellos es dependiente del regulador de respuesta OmpR. Análisis por protección a DNasa I mostraron que *ompS1* posee seis cajas de unión para OmpR, donde principalmente las cajas I-II-III que están cercanas al promotor, mostraron alta afinidad para el regulador. El promotor P2, a diferencia de P1, no requiere a OmpR para su activación, al contrario, solamente es activo en la ausencia de OmpR, ya que OmpR reprime este promotor uniéndose entre la caja -10 y -35 (Oropeza *et al*., 1999).

2.3 H-NS silencia *ompS1*

Usando un sistema de mutagénesis al azar por transposón, se aislaron mutaciones que desreprimieron la expresión de *ompS1* tanto en *S*. Typhi como en *S*. Typhimurium. Estas mutantes mapearon en el gen *hns*, específicamente hacia el 3' en la región que codifica para el carboxilo terminal de la proteína H-NS (dominio de unión a DNA). Una mutante *hns* en *S*. Typhi (STY*hns*99) desreprimió la expresión de *ompS1* aproximadamente 10 veces comparada a la cepa silvestre (IMSS-1) (Fig. 11; Flores-Valdez *et al*., 2003).

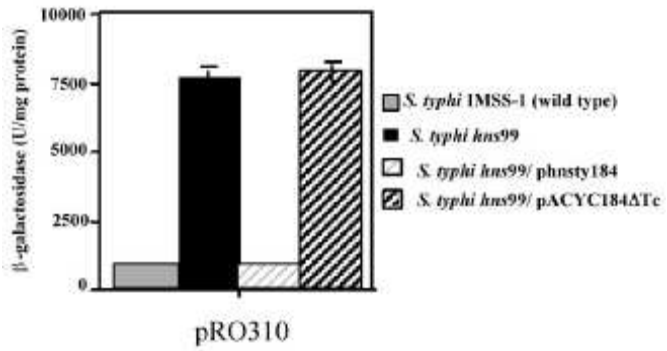

Fig. 11. H-NS negativamente regula la expresión de *ompS1*. La fusión pRO310, que es la más larga y contiene todos los elementos de regulación de *ompS1*, fue desreprimida en una mutante *hns* (STY*hns*99). Esta mutante complementada con un plásmido que contiene el gen *hns*, restableció la expresión de *ompS1* similar a la cepa silvestre. Imagen tomada de Flores-Valdez *et al*., 2003.

Por medio de ensayos en cambio en la movilidad electroforética (EMSA, por sus siglas en inglés) se encontró que H-NS regula directamente la expresión de *ompS1* uniéndose específicamente a su región reguladora (310 pb) (Fig. 12). En esta región se encontraron zonas con curvaturas intrínsecas significativas hacia el 5' (Flores-Valdez *et al*., 2003).

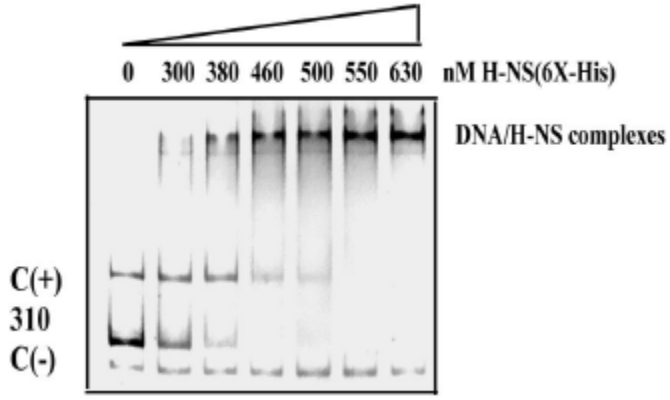

Fig. 12. H-NS se une directamente a la región reguladora de *ompS1*. Ensayos tipo EMSA fueron realizados incubando diferentes concentraciones de la proteína H-NS con la región reguladora de *ompS1* (310 pb). Como control positivo y negativos fueron usadas las regiones reguladoras y codificantes del gen *ler* de EPEC, respectivamente. Imagen tomada de Flores-Valdez *et al*., 2003.

Para evaluar el efecto de la interrupción de *hns* sobre la expresión de *ompS1* en *S*. Typhi, se realizaron ensayos de "primer extensión" (PE). La expresión de *ompS1* cromosomalmente no se detectó ni el fondo silvestre ni en ausencia de OmpR, sin embargo, en la mutante *hns*99 su expresión si se detectó comparada a la cepa silvestre proviniendo del promotor P1 (Fig. 13). En la doble mutante *hns*99 Δ*ompR*, pudo detectarse la actividad del promotor P2, a comparación de la mutante sencilla Δ*ompR* (Fig. 13). Estos resultados indicaron que la proteína H-NS reprimió ambos promotores de *ompS1* (P1 y P2) (Flores-Valdez *et al*., 2003).

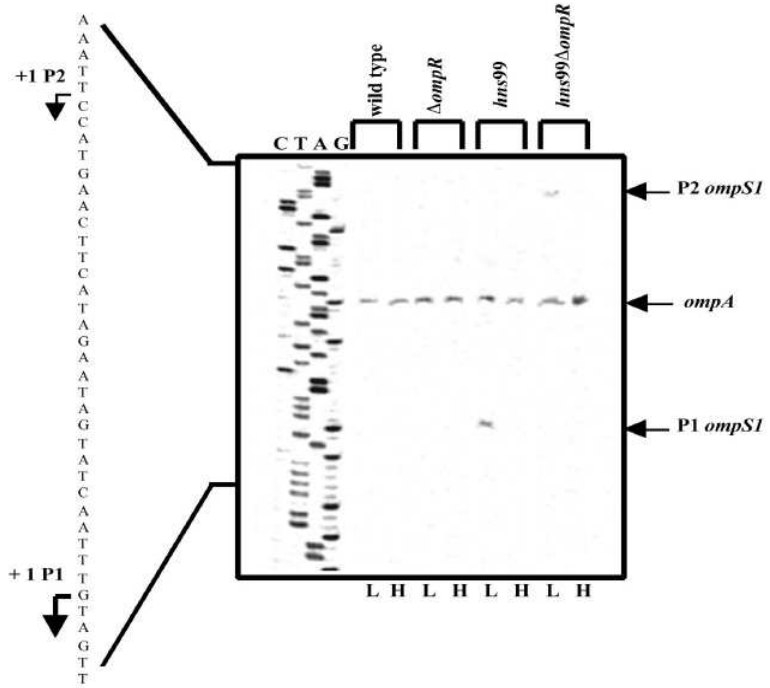

Fig. 13. Actividad de los promotores de *ompS1* bajo la ausencia de H-NS y OmpR. Ensayo tipo Primer Extension fueron realizados en baja (L) y alta (H) osmolaridad usando RNA cromosomal de varios fondos genéticos: wt, Δ*ompR*, *hns*99 y *hns*99 Δ*ompR*. El promotor P1 fue detectado en el fondo *hns*, mientras que el promotor P2 fue detectado en ausencia de H-NS y OmpR. Imagen tomada de Flores-Valdez *et al*., 2003.

3. HIPÓTESIS

Además de H-NS, existe otro elemento de regulación negativa que reprime la expresión de *ompS1* en un fondo STY*hns*99. Existe un programa en la célula que contrarresta el silenciamiento ejercido sobre *ompS1*. Asimismo, el promotor P2 de *ompS1* es activado por un regulador transcripcional diferente a OmpR.

4. OBJETIVOS

General:

- Analizar los mecanismos moleculares que regulan la expresión de *ompS1* en *Salmonella enterica* serovar Typhi.

Específicos:

- Determinar elementos de regulación positiva (además de OmpR) involucrados en la activación de *ompS1*.

- Identificar otros represores (además de H-NS) que silencian la expresión de *ompS1*.

- Evaluar el mecanismo de regulación mediada por el concierto de reguladores para controlar el apagado y encendido del gen *ompS1*.

5. MATERIALES Y MÉTODOS

Los materiales y métodos que se describirán en esta sección, son aquellos que no aparecen en los trabajos publicados. En la tabla 2 se presenta una lista de cepas y plásmidos usadas en este trabajo. También se describirán las metodologías no publicadas.

Tabla 2. Lista de cepas y plásmidos

Cepas o plásmidos	Genotipo y/o marcadores relevantes	Referencia
***S.* Typhi**		
IMSS-1	*Salmonella enterica* serovar Typhi, cepa silvestre de referencia clínica.	Puente *et al*., 1987
IMSS-40	IMSS-1 Δ*ompR*::Km^r	Fernández-Mora *et al*., 2004
IMSS-50	IMSS-1 Δ*cpxR*::Km^r	Este trabajo
IMSS-60	IMSS-1 Δ*ompR* Δ*cpxR*::Km^r	Este trabajo
IMSS-81	IMSS-1 Δ*ompB*::Km^r	Martínez-Flores *et al*., 1999
Plásmidos		
pRO310	pMC1871 conteniendo una fusión traduccional a *lacZ* de la región reguladora de *ompS1* de -310 a +27 pb con respecto a P1.	Oropeza *et al*., 1999
pRO88	pMC1871 conteniendo una fusión traduccional a *lacZ* de la región reguladora de *ompS1* de -88 a +27 pb con respecto a P1.	Oropeza *et al*., 1999
pSCZ10	pMC1871 conteniendo una fusión traduccional a *lacZ* de la región reguladora de *ompC* de -1450 al codón de inicio con respecto a P1.	Martínez-Flores *et al*., 1999
pCA-CpxR-GFP	pCA24N llevando el gen estructural de *cpxR* fusionado a 6XHis en el amino terminal y la proteína GFP en el carboxilo terminal;	Kitagawa *et al*., 2005
pFM2000	pACYC184 llevando el gen *ompR* de *S.* Typhi; p15A1.	Fernández-Mora *et al*., 2004
pFVD55A	pMPM-A3 llevando el gen *ompR* de *S.* Typhi con una mutación puntual en Asp-55 (D55A); p15A1.	Este trabajo
pFM*TrcleuO*-50	pFM*Trc12* llevando el gen *leuO* de *S.* Typhi fusionado a 6XHis en el amino bajo el promoter *trc*; p15A1.	De la Cruz *et al*., 2007

Generación de mutantes *cpxR* y *ompR cpxR*

Las mutantes sencillas y dobles en los genes *ompR* y *cpxR* fueron generadas por la metodología reportada por Datsenko and Wanner 2000, usando la cepa silvestre IMSS-1. Los oligonucléotidos usados para amplificar la resistencia a Kanamicina y que llevaron secuencias homólogas al gen *cpxR* de *S*. Typhi fueron: 5'-GAT GAC CGA GAG CTG ACT TCC CTG TTA AAA GAG CTC CTC GAA TGT AGG CTG GAG CTG CTT CG-3' (H1P1), y 5'-TGT TTT AAA CCA CGG GTG ACC GTC TTT GCG TTC CGG CAG TTT CAT ATG AAT ATC CTC CTT AG-3' (H2P2). La doble mutante Δ*ompR* Δ*cpxR* se generó a partir de la mutante sencilla Δ*ompR* (Fernández-Mora *et al*., 2004) a la cual se le quitó la resistencia a kanamicina usando el plásmido pCP20 para poder seleccionar. Ambas mutantes fueron caracterizadas por perfil de proteínas de membrana externa y por PCR.

Anisotropía de fluorescencia

Las mediciones de unión en el equilibrio fueron hechas usando el protocolo reportado por Head *et al*., 1998. Se usaron oligonucleótidos complementarios que abarcan la región promotora de *ompS1* (71 pb), específicamente las cajas I-II-III de unión a OmpR. Uno de los oligonucleótidos que forma la doble cadena fue marcado con fluoresceína. El cambio en la anisotropía, donde ΔF/Fo representa la diferencia en anisotropía en la presencia de la proteína menos la anisotropía en la ausencia de la proteína dividida por la anisotropía en la ausencia de la proteína, se grafica como una función de la concentración total de la proteína. Los resultados de las curvas de unión fueron ajustadas por una regresión cuadrada no lineal como fue descrita previamente (Head *et al*., 1998). La constante de disociación aparente reportada en el texto representa la media.

ELISA

Se usaron oligonucléotidos complementarios para formar una doble cadena de DNA (66 pb). Estos oligonucleótidos abarcaron la región promotora de *ompS1*, conteniendo específicamente una secuencia consenso encontrada para CpxR. Uno de los oligonucleótidos fue acoplado a biotina en el 3'. Se usaron placas de 96 pozos acopladas a estreptavidina. La doble cadena se formó calentado los oligonucléotidos complementarios a 95^{o}C por 3 minutos, dejando bajar la temperatura lentamente hasta llegar a la del ambiente. Esta doble cadena se incubó en los pozos acoplados a

estreptavidina por 1 hora. Se hicieron lavados con PBS 1X (3 veces) y posteriormente se agregó leche descremada al 5 % y se incubó durante 1 hora. Se lavaron los pozos con PBS 1X y posteriormente se incubaron cantidades crecientes de la proteína CpxR-GFP en los pozos por 1 hora. Se lavaron los pozos con PBS 1X y se agregó el anticuerpo primario anti-GFP (1:10,000) por 1 hora. Se lavaron los pozos con PBS 1X y se agregó el anticuerpo secundario acoplado a peroxidada (HRP) por 1 hora (1:10,000). Posteriormente se agregó el reactivo, cuya reacción fue medida por ELISA. Los datos fueron graficados en una curva que era una función de la absorbancia y la concentración de la proteína.

6. RESULTADOS

6.1 LeuO contrarresta la represión ejercida por H-NS y StpA sobre *ompS1*

Además de H-NS, analizamos el efecto represor de StpA sobre la expresión de *ompS1*. En un fondo *hns* la expresión de *ompS1* se desreprimió pero no alcanzó los máximos niveles comparados a la fusión pRO88. Una mutante sencilla *stpA* mostró los mismos niveles de represión comparados a la cepa silvestre. Interesantemente en un fondo *hns stpA*, los niveles de OmpS1 en la membrana externa fueron encontrados abundantes. La expresión de *ompS1* fue positivamente regulada por LeuO, un regulador quiescente tipo LysR. Cuando se induce el gen *leuO* en la cepa silvestre, la expresión de *ompS1* fue completamente desreprimida y la porina OmpS1 fue detectada abundantemente en la membrana externa. LeuO activó ambos promotores de *ompS1* (P1 y P2) y se unió secuencias arriba (-135 a -100) que se sobreponen a un sitio de nucleación de H-NS (-110 a -104). Estos resultados son consistentes con un mecanismo donde LeuO actuó como una antagonista de H-NS, desplazándolo de la región reguladora de *ompS1.*

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LeuO antagonizes H-NS and StpA-dependent repression in *Salmonella enterica ompS1*

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Summary

The *ompS1* gene encodes a quiescent porin in *Salmonella enterica*. We analysed the effects of H-NS and StpA, a paralogue of H-NS, on *ompS1* expression. In an *hns* single mutant expression was derepressed but did not reach the maximum level. Expression in an *stpA* single mutant showed the same low repressed level as the wild type. In contrast, in an *hns stpA* background, OmpS1 became abundant in the outer membrane. The expression of *ompS1* was positively regulated by LeuO, a LysR-type quiescent regulator that has been involved in pathogenesis. Upon induction of the cloned *leuO* gene into the wild type, *ompS1* was completely derepressed and the OmpS1 porin was detected in the outer membrane. LeuO activated the P1 promoter in an OmpR-dependent manner and P2 in the absence of OmpR. LeuO bound upstream of the regulatory region of *ompS1* overlapping with one nucleation site of H-NS and StpA. Our results are thus consistent with a model where H-NS binds at a nucleation site and LeuO displaces H-NS and StpA.

Introduction

Salmonella enterica serovar Typhi (*S.* Typhi) is the aetiological agent of typhoid fever in humans (Pang *et al.*, 1998). *S.* Typhi synthesizes three major outer membrane proteins (OMPs), OmpC, OmpF and OmpA. OmpC and OmpF in *S.* Typhi are under the control of the EnvZ-OmpR two-component system as in *Escherichia coli* (Puente *et al.*, 1991). In our laboratory we have identified the *S. enterica ompS1* and *ompS2* genes that code for the OmpS1 and OmpS2 quiescent porins that belong to the OmpC/OmpF superfamily (Fernández-Mora *et al.*, 1995; 2004; Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). OmpS1 appears to have a role in swarming motility, biofilm formation and virulence in mice (Toguchi *et al.*, 2000; Mireles *et al.*, 2001; Rodríguez-Morales *et al.*, 2006).

Expression of *ompS1* is dependent on two overlapping promoters, P1 and P2. The P1 promoter is dependent on the OmpR response regulator: the *ompS1* upstream regulatory region possesses six OmpR binding sites. The P2 promoter does not require OmpR for activation and is only active in the absence of OmpR. The regulatory region between positions –310 to +27 with respect to the transcriptional start site of P1 contains both positive and negative regulatory elements. Sequences upstream of position –88 are required for negative regulation and sequences downstream of –88 are required for positive regulation (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003).

H-NS, a nucleoid protein of 137 amino acids (~15 kDa), negatively regulates *ompS1* expression by binding upstream of position –88 (Flores-Valdez *et al.*, 2003). In *Salmonella*, H-NS has been proposed to selectively silence horizontally acquired genes by targeting sequences with GC content lower than the resident genome, regulating around 12% of its genes (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). *E. coli* and other related enteric bacteria such as *S. enterica* contain StpA, an H-NS paralogue (Shi and Bennett, 1994; Zhang *et al.*, 1996; Bertin *et al.*, 2001; Tendeng and Bertin, 2003; Madrid *et al.*, 2007). StpA binds both to RNA and DNA, and also shows preference for curved DNA sequences (Zhang *et al.*, 1996; Sonnenfield *et al.*, 2001). Analysis of *stpA* and *hns* expression has indicated that the two gene products are capable of both negative autogenous control and cross-regulation; that is, StpA levels are significantly increased in an *hns* mutant background. Because of the high level of amino acid sequence identity between H-NS and StpA (58%), it has been proposed that StpA is a molecular back-up for H-NS (Shi and Bennett, 1994; Sondén and Uhlin, 1996; Zhang *et al.*, 1996; Sonnenfield *et al.*, 2001).

LeuO is a LysR-type quiescent regulator that is under negative control by H-NS (Klauck *et al.*, 1997; Chen *et al.*,

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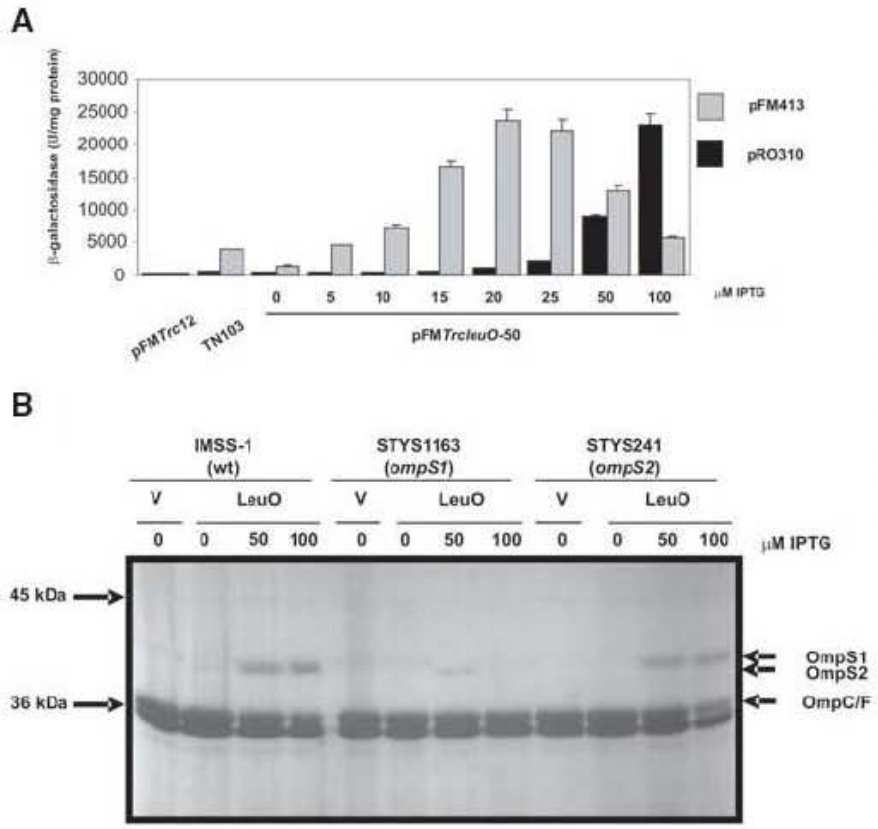

Fig. 1. LeuO as a positive regulator of *ompS1* and *ompS2* in *S.* Typhi.
A. β-Galactosidase activity of *ompS1–lacZ* (pRO310) and *ompS2–lacZ* (pFM413) gene reporter fusions in the presence of the plasmid pFM*TrcleuO*-50, induced under a gradient of IPTG (0–100 μM) in *S.* Typhi wild type (wt). The activities by vector plasmid pFM*Trc12* were used as negative control; in the mutant strain IMSSTN103 (Fernández-Mora *et al.*, 2004).
B. Outer membrane protein preparations showing expression of OmpS1 in the presence of LeuO (pFM*TrcleuO*-50) under different concentrations of IPTG, wild type (IMSS-1), *ompS1* (STYS1163) and *ompS2* (STYS241) mutants. The effect of vector plasmid V (pFM*Trc12*) is shown as negative control. OmpS2 can be observed when LeuO is induced at 50 μM IPTG in the *ompS1* mutant.

2005; Navarre *et al.*, 2006). It has been implicated in the bacterial response to stress (VanBogelen *et al.*, 1996; Fang *et al.*, 2000; Majumder *et al.*, 2001), in virulence (Tenor *et al.*, 2004; Lawley *et al.*, 2006; Rodríguez-Morales *et al.*, 2006) and in biofilm accumulation (Moorthy and Watnick, 2005). Furthermore, expression of LeuO has been found to affect the expression of several genes influenced by mutations in *hns*: LeuO relieved silencing of the *bgl* operon, the operon involved in the utilization of certain β-glucosides (Ueguchi *et al.*, 1998); suppressed the effect of an *hns* mutation on *cadC*, which codes for the activator of the acid-inducible lysine decarboxylase (Shi and Bennet, 1995); and also suppressed the negative effect of the AT4 region, the 72 bp gene silencer region located between the *leuO* gene and the *leuABCD* leucine biosynthetic operon, by delimiting the transcriptional repression by H-NS through the binding of LeuO to the promoter region (Chen *et al.*, 2001; 2003). Besides, LeuO has a negative effect on the expression of the small regulatory DsrA RNA that increases sigma factor RpoS translation (Klauck *et al.*, 1997). It has been reported more recently that LeuO positively regulates the expression of *rovA*, which codes for the key regulator of virulence in *Yersinia* (Lawrenz and Miller, 2007).

Interestingly, we had shown previously that LeuO positively regulates *ompS2* (Fernández-Mora *et al.*, 2004). We report here that LeuO and the silencing proteins H-NS and StpA are the main positive and negative regulators of *ompS1* expression in *Salmonella*. LeuO positively regulated the *ompS1* expression by derepressing both promoters and antagonizing the negative effect of H-NS and StpA, displacing them from the 5′ regulatory region. In this study, LeuO was found to bind upstream and not to interact with the *ompS1* promoter region.

Results

LeuO positively regulated ompS1 *expression in* Salmonella enterica

We have reported previously that LeuO positively regulates *ompS2* expression in *S.* Typhi (Fernández-Mora *et al.*, 2004). To test whether LeuO regulated the *ompS1* gene, LeuO was expressed in *S.* Typhi wild type from plasmid pFM*TrcleuO*-50 induced with a gradient of IPTG concentrations (0–100 μM), and the effect was determined on the expression of *S.* Typhi *ompS1–lacZ* (pRO310) and *ompS2–lacZ* (pFM413) gene reporter fusions (Oropeza *et al.*, 1999; Fernández-Mora *et al.*, 2004) (Fig. 1A). Interestingly, the pRO310 *ompS1–lacZ* fusion was also positively regulated by LeuO, but at higher concentrations of IPTG than previously reported for *ompS2*. The highest level of *ompS1* expression

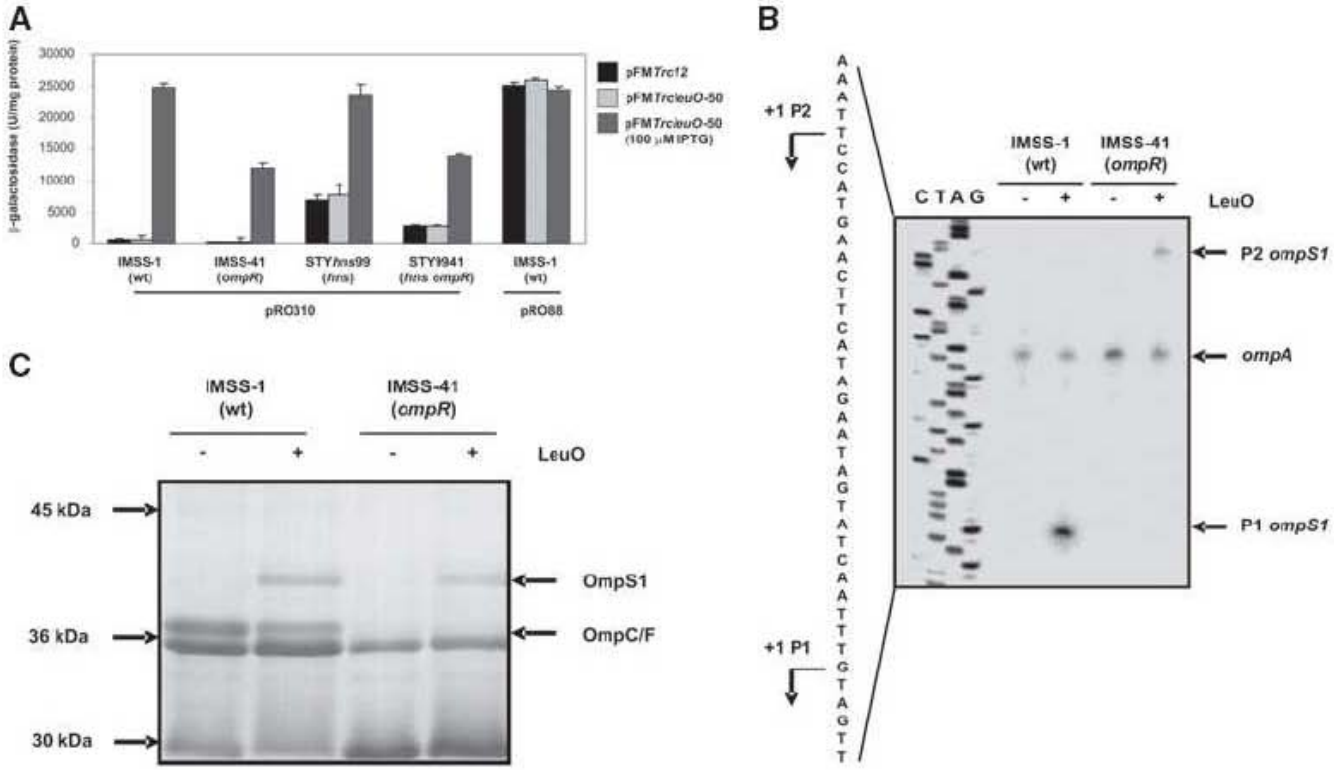

Fig. 2. LeuO positively regulates both *ompS1* promoters.
A. β-Galactosidase activity of the *ompS1–lacZ* (pRO310) fusion in *S.* Typhi wild type (wt), and in the isogenic *ompR, hns* and *hns ompR* mutants, in the presence of the pFM*Trc12* vector plasmid, the pFM*TrcleuO*-50 plasmid with and without induction (100 μM IPTG) were evaluated.
B. Primer extension analysis of the *ompS1* P1 and P2 promoters from the chromosomal gene in *S.* Typhi wild type and in the *ompR* mutant, in the presence of pFM*TrcleuO*-50 plasmid induced (+) or not (–) at 100 μM IPTG. The *ompA* transcript was included as a loading control.
C. Outer membrane protein profiles of *S.* Typhi wild type and *ompR* mutant, in the presence of the pFM*TrcleuO*-50 plasmid induced at 100 μM IPTG (+) or of the pFM*Trc12* plasmid vector (–).

(–25 000 U) was obtained when LeuO was induced at 100 μM IPTG. It is interesting that at the highest levels of LeuO induction *ompS1* expression increased whereas *ompS2* expression decreased. In contrast, the level observed for *ompS1* expression was constant at the higher concentrations of IPTG up to 1 mM (data not shown).

Expression of pRO310 and pFM413 is also shown in the original *S.* Typhi *Tn*-10 insertion mutant (IMSSTN103), where the transposon insertion promoted *leuO* expression and allowed us to identify LeuO as a positive regulator of *ompS2* (Fernández-Mora *et al.*, 2004). As observed, *ompS1* expression was not induced as compared with the wild type, and only *ompS2* expression could be readily detected albeit at a relatively low level.

OmpS1 could now be detected as an OMP in the *S.* Typhi wild type, upon induction with LeuO from pFM*TrcleuO*-50 at 50 and 100 μM IPTG (Fig. 1B). The identity of the OmpS1 band in the Δ*ompS2* (STYS241) mutant strain was verified by mass spectrometry analysis. Hence, LeuO regulated positively the *ompS1* gene.

LeuO derepressed both ompS1 *promoters*

To elucidate how LeuO, OmpR and H-NS regulate *ompS1* expression, the effect of LeuO was evaluated in the wild type (IMSS-1), in the *ompR* mutant (IMSS-41), in the *hns* mutant (STY*hns*99) and in the *hns ompR* double mutant (STY9941). The STY*hns*99 mutant was obtained upon random mutagenesis of the *S.* Typhi genome, in the search for *ompS1*-negative effectors (Flores-Valdez *et al.*, 2003).

The expression value levels for *ompS1* at 100 μM IPTG in the wild type were –25 000 U (Fig. 2A). This maximum level of expression is as observed for the pRO88 fusion that lacks the *cis*-negative regulatory elements (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). In the *ompR* mutant the expression was induced to –12 000 U (Fig. 2A), indicating the importance of OmpR in order to reach the maximum level of *ompS1* expression. Furthermore, expression in the *hns* mutant derivatives increased in the presence of LeuO, indicating that it can derepress *ompS1* expression to the maximum level. In the *hns ompR* double mutant, the levels of expression were half of

those observed in the *hns* single mutant upon induction of LeuO (Fig. 2A).

Because of the fact that *ompS1* has two promoters, primer extension experiments were performed in order to assess the effect of LeuO on each promoter from the chromosomal gene. When LeuO was induced in the wild type, expression was observed only from P1; whereas in the *ompR* mutant the expression came from P2 (Fig. 2B). This further illustrates how OmpR differentially regulates both promoters (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). Figure 2C depicts the presence of OmpS1 in the outer membrane upon induction with LeuO, even in the *ompR* mutant (under P2). It is thus evident that OmpR is required only for the highest levels of expression, and that it determines the use of the promoters thus acting as a modulator of the *ompS1* expression; and that LeuO is the main regulator by derepressing both P1 and P2.

StpA repressed ompS1 *expression in a mutant* hns *background*

H-NS negatively regulates *ompS1* expression in both *S.* Typhi and *S.* Typhimurium (Flores-Valdez *et al.*, 2003). Moreover, the pattern of *S.* Typhi *ompS1* regulation is similar in *S. enterica* and in *E. coli* (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). However, as the expression of the *ompS1–lacZ* pRO310 fusion was still partially repressed in a *Salmonella hns* mutant (Flores-Valdez *et al.*, 2003; Fig. 2A), the possibility of StpA also acting as a repressor was explored as StpA is increased in a mutant *hns* background (Sondén and Uhlin, 1996; Zhang *et al.*, 1996; Sonnenfield *et al.*, 2001). Given that null mutations in *hns* are lethal in *S. enterica* unless accompanied by compensatory mutations in *rpoS* or *phoP* (Navarre *et al.*, 2006), and as we were unable to generate a double *hns stpA* mutant in *S. enterica*, the effect of H-NS and StpA in *S.* Typhi was assessed using a dominant negative system that codes for an H-NS protein with a mutation in the C-terminal domain (G113D) (Ueguchi *et al.*, 1996). This mutant is defective in the ability to repress transcription but not in the ability to interact with other H-NS monomers. The negative dominant allele was cloned in a pMPM-T6Ω vector that is inducible by arabinose (pT6-HNS-G113D) (Bustamante *et al.*, unpubl. data).

We tested two *ompS1* fusions, pKFV310 and pKFV88, being equivalent to pRO88 and pRO310, respectively, but with the appropriate antibiotic resistance marker (pROs and pT6-HNS-G113D are Tc^r). The *ompS1* expression was determined under the induction of pT6H-NSG113D in three *S.* Typhi backgrounds: wild type (IMSS-1), *hns* mutant (STY*hns*99) and *stpA* mutant (STY Δ*stpA*) (Fig. 3A). In the wild type, the induction of pT6-HNS-G113D resulted in the derepression of *ompS1* expression,

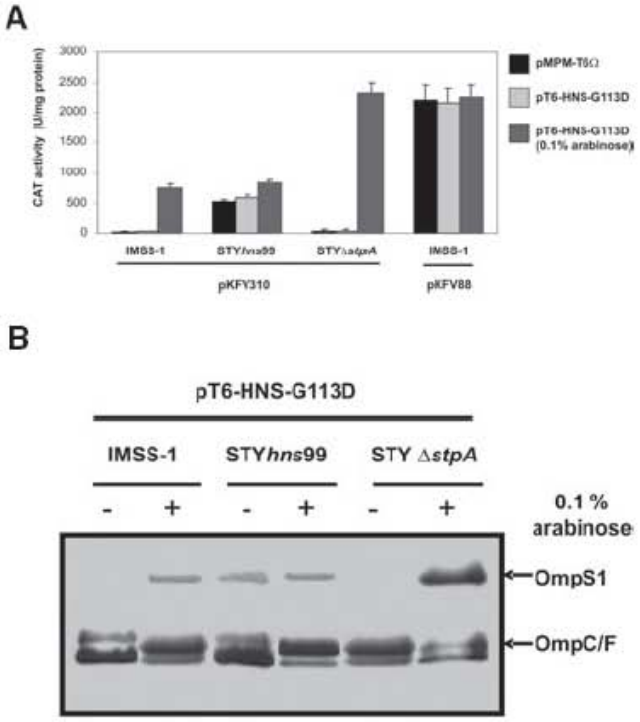

Fig. 3. StpA also acts as a silencer for *ompS1* expression in addition to H-NS.
A. CAT activity of the pKFV310 fusion in *S.* Typhi wild type, in an *hns* and *stpA* mutants in the presence of pMPM-T6Ω vector plasmid, the pT6-HNS-G113D (an *hns*-negative dominant allele) with and without induction (0.1% arabinose) were evaluated. The pKFV88 *ompS1-cat* reporter is shown as control.
B. Outer membrane protein profiles of *S.* Typhi wild type and mutants harbouring plasmid pT6H-NSG113D induced (+) or not (–) with 0.1% arabinose.

reaching the levels of an *hns* mutant (Fig. 3A). The induction of pT6H-NS-G113D had no significant effect on *ompS1* expression in the *hns* background but, interestingly, in the *stpA* background the levels of *ompS1* expression were maximum being similar to the pKFV88 fusion that lacks the negative regulatory elements (Fig. 3A). These observations were corroborated determining the presence of OmpS1 in the outer membrane, coded by the *S.* Typhi chromosomal gene, expressed upon induction of the negative dominant allele. OmpS1 was detected in the wild type and in the *hns* background, and in the *stpA* mutant it was very abundant as expected (Fig. 3B). Our results indicate that indeed H-NS and StpA silence *ompS1* expression in *S.* Typhi, where StpA represses only in an *hns* background.

H-NS, StpA and LeuO bound around OmpR box IV

H-NS was found to bind upstream of position –88 of *ompS1* P1 (Flores-Valdez *et al.*, 2003). To determine more precisely the site for H-NS binding, several fragments from the *S.* Typhi *ompS1* regulatory region were

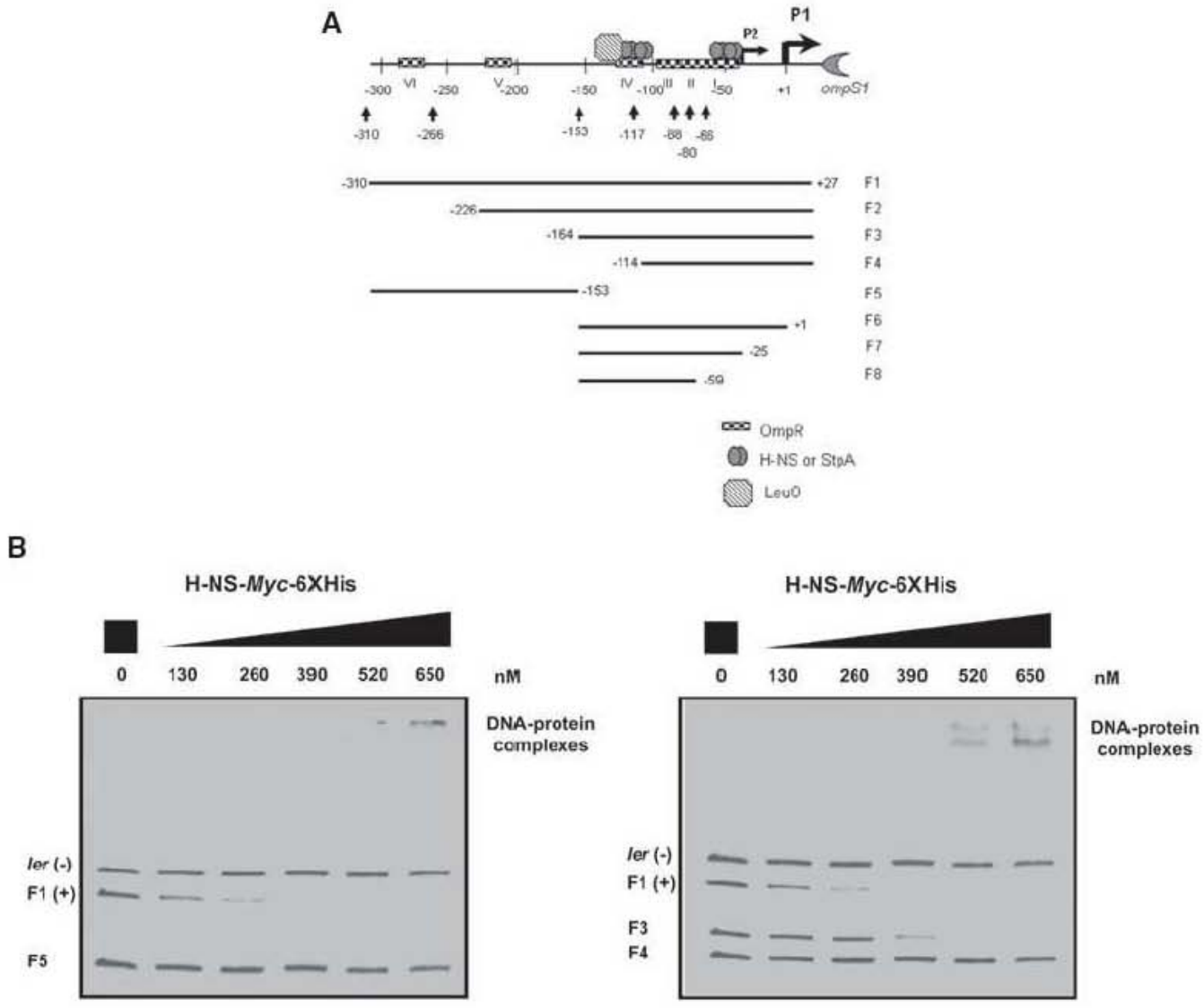

Fig. 4. H-NS, StpA and LeuO bind around OmpR box IV.
A. Schematic representation of the *ompS1* regulatory region showing the +1 transcribed nucleotide at P1, OmpR-dependent promoter, the P2 promoter and the upstream bp co-ordinates. Also shown is the position for the various DNA lengths in a collection of gene *lacZ* reporter fusions (arrows) and for the binding of OmpR (Oropeza *et al.*, 1999), as well as the binding of H-NS or StpA, and LeuO according to this study (panels B, C and D). The PCR F-fragments used for the electrophoretic mobility shift assays (EMSAs) are depicted.
B. EMSA exemplifying the binding of various concentrations of H-NS to a positive F1 control fragment and to a negative *ler* control fragment as compared to the binding to F3, F4 and F5 fragments.
C. EMSA showing the binding of various concentrations of H-NS and StpA to different F-fragments.
D. DNase footprinting analysis showing the binding of several concentrations of H-NS to OmpR box IV. The vertical dark bar indicates the protected regions and the vertical light line shows the mutated site in bIV–mt2 (Fig. 5A).
E. EMSA showing the binding of various concentrations of LeuO to different F-fragments.
F. DNase footprinting analysis showing the binding of various concentrations of LeuO (0–1 μM) to OmpR box IV. The vertical dark bars indicate the protected regions, the rectangle the common motif for LeuO-binding boxes and the vertical grey bar indicates the DNase hypersensitive region.

amplified by polymerase chain reaction (PCR; Fig. 4A, Table 1) and subjected to an electrophoretic mobility shift assay (EMSA). Each fragment was evaluated using as negative control, the *ler*-coding region of enteropathogenic *E. coli* and as positive control, the *ompS1* regulatory region (F1); both controls previously had been characterized by Flores-Valdez *et al.* (2003), as exemplified in Fig. 4B. Thus, H-NS bound to fragment F1 that encompasses the whole 310 bp regulatory region, as expected (Fig. 4A–C); but not to the region upstream of –153 (F5). Moreover, H-NS bound to F2 and F3 but not to F4 (Fig. 4C), indicating that a binding site mapped between –164 and –114, overlapping OmpR-binding box IV. Moreover, the removal of sequences upstream of box IV

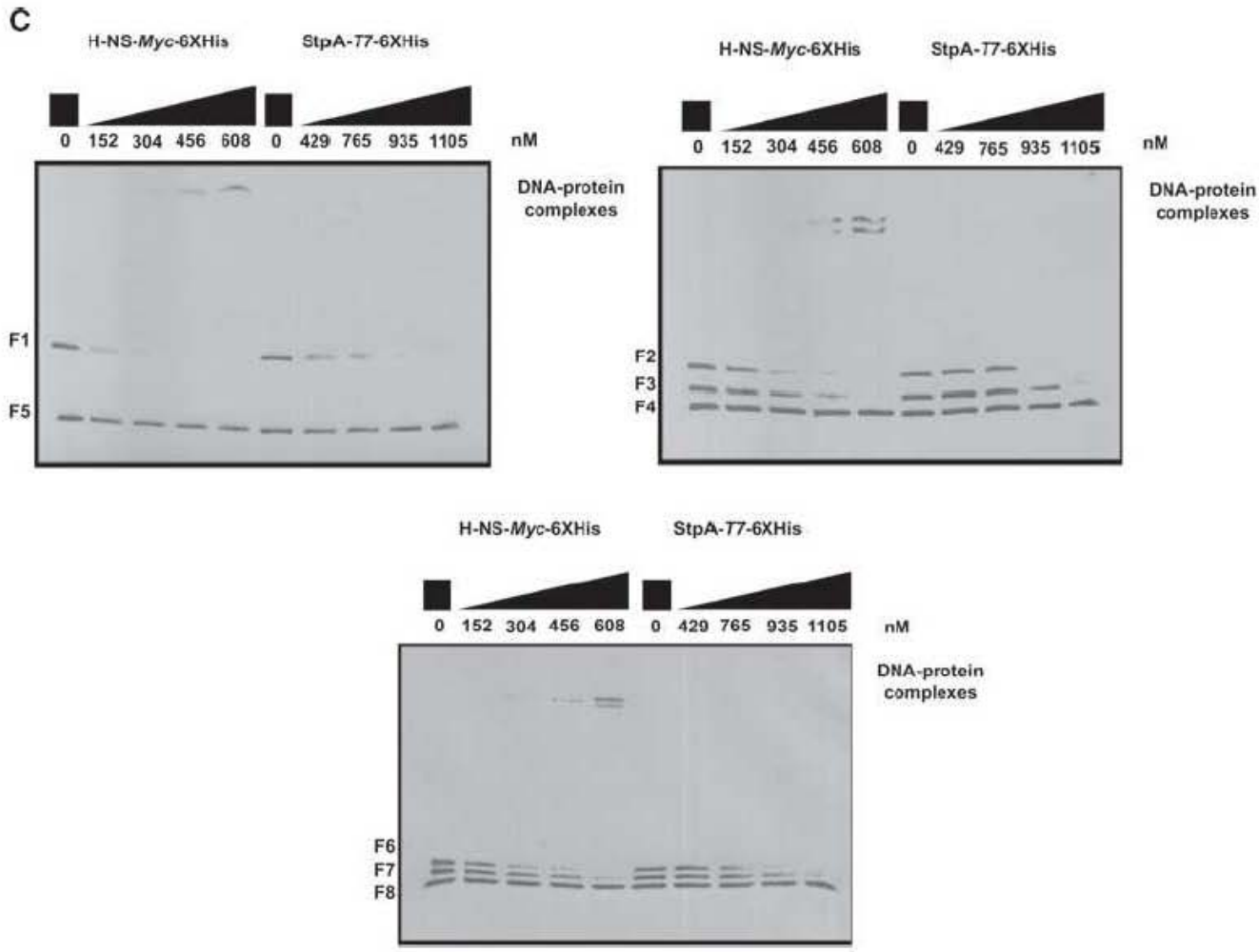

Fig. 4. *cont.*

caused a diminished affinity to H-NS, showing their relevance for efficient binding (Fig. 4C), as illustrated by F2 as compared with F3.

On the other hand, the binding to fragments F6 and F7 and not to F8 indicated a second binding site at –59 to –25 (Fig. 4A and C). In terms of StpA, it bound to the same DNA fragments, but at twofold to threefold higher concentrations than H-NS (Fig. 4A and C). As the presence of both sites was needed for H-NS and StpA interaction, i.e. there was no binding to fragments F4 nor F8 that lack one of the two sites, we postulate that both of them are required for proper DNA binding (Fig. 4A and C).

DNase footprinting was performed to determine the binding of H-NS to the *ompS1* regulatory region in the vicinity of OmpR-binding box IV (–70 to –200 bp upstream

Table 1. Oligonucleotide primers used for generating various *ompS1* fragments from the 5′ regulatory region by PCR for EMSA.

Primer	Sequence	Alignment	Reference
310b-1	5′-TAGCCTTTTATCATTTATTTTATC-3′	–303 Fwd	Flores-Valdez *et al.* (2003)
310b-2	5′-ATGAGTTATGTGTTTTGATTTGA-3′	–153 Rev	Flores-Valdez *et al.* (2003)
310b-3	5′-CAAAGCATCAAATACATATAAAAA-3′	–226 Fwd	Flores-Valdez *et al.* (2003)
310b-4	5′-TGTTTCTATTTGGTTTTTTATAATAC-3′	–76 Rev	Flores-Valdez *et al.* (2003)
310b-5	5′-GAATATATAGTCTATTATTATCATTTT TTATGAATGAGTTATGTG-3′	–164 Fwd	This study
310b-7	5′ATTCTTAGTCACTTATATATCCTGTAT TAT-3′	–114 Fwd	Flores-Valdez *et al.* (2003)
310b-8	5′-AATATGTAGCCACTTCAACAAAAC-3′	+27 Rev	Flores-Valdez *et al.* (2003)
310-(+1)	5′-CTACAAATTGATACTATTCTATG-3′	+1 Rev	This study
310-(P2)	5′-CATGGAATTTAAAAAATAACATG-3′	–25 Rev	This study
310-(BII)	5′-TTTAAAATATTTCAATTTGTTTC-3′	–59 Rev	This study

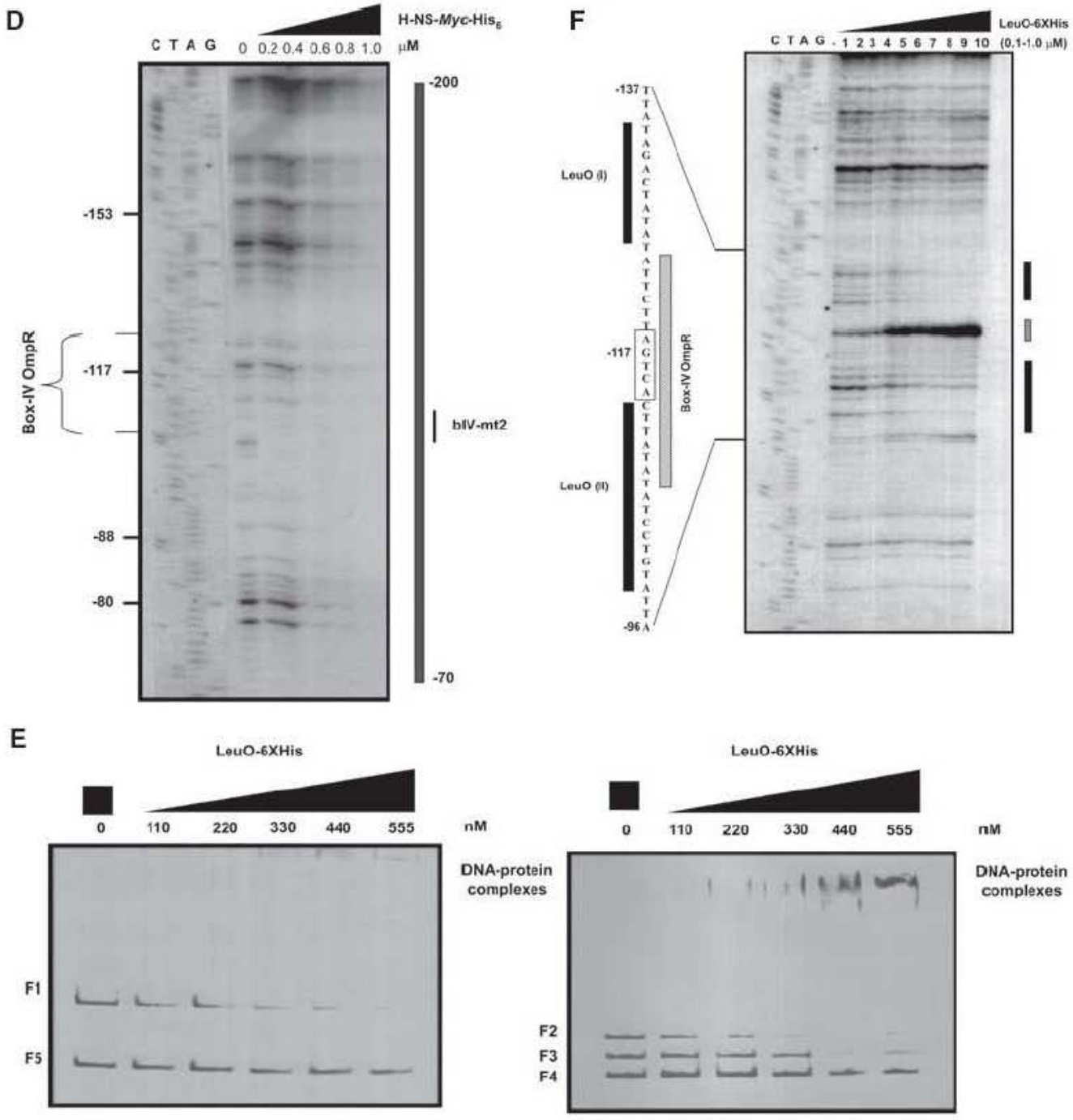

Fig. 4. *cont.*

of the P1 transcriptional start). As can be observed, H-NS bound indeed around OmpR-binding box IV at 0.2 and 0.4 µM (Fig. 4D) corroborating the data generated by EMSA (Fig. 4C). Upstream and downstream sequences of OmpR-binding box IV were protected at higher H-NS concentrations (0.6–1.0 µM).

LeuO also bound downstream of –153 as shown by the binding to F1 and not to F5 (Fig. 4A and E). Furthermore, it bound to F2, F3, but not to F4, indicating that it also bound between positions –164 and –114 (Fig. 4A and E). No other region for LeuO binding was detected as for H-NS and StpA (not shown). The binding site for LeuO was further defined by a DNase footprinting analysis between positions –137 and –96 (Fig. 4F). Specifically, LeuO bound at a region with two sites: one between –134 and –125, LeuO (I), and another at –113 to –99, LeuO (II), where the first site generated greater protection by LeuO than the second (Fig. 4F). As can be appreciated, the

contact points for LeuO overlap OmpR-binding box IV and the –164 to –114 binding site for H-NS and StpA (Fig. 4A, C and D). Interestingly, the LeuO binding site shared the ANTCA motif with the binding site at *ompS2* (Fernández-Mora *et al*., 2004) and *leuO* (Chen *et al*., 2003); and with a site required for LeuO activity on *dsrA* (Repoila and Gottesman, 2001). A DNase hypersensitive site between the contact points developed upon the binding of LeuO, indicating a restructuring in the DNA fold (Fig. 4F).

These data showed that the binding of H-NS and StpA was located at two sites and that LeuO bound upstream from the *ompS1* regulatory region, overlapping with one binding site of H-NS and StpA, but not interacting with the promoter region and promoting a change in the local DNA architecture.

cis-*acting site required for LeuO derepression*

To demonstrate by genetic means the relevance of the LeuO binding region in the derepression of *ompS1,* we generated a series of point mutants in the interaction site of the protein in agreement with the DNase footprinting assay. Thus, site-directed mutagenesis was performed on the pRO310 reporter plasmid (Fig. 5A). The pRO310 bIV–mt1 mutant carried mutations on LeuO (I) (–134 to –125), the site with greater protection for LeuO (Fig. 4F). The changes were A to T and T to A, preserving the G and C residues. The pRO310 bIV–mt2 mutant carried mutations on LeuO (II) (–113 to –102) modifying A to T (Fig. 5A). The expression of the mutants was evaluated under the absence or presence of LeuO induction. The pRO310 bIV–mt1 fusion resulted in the lack of activity under LeuO induction with respect to the wild type (Fig. 5B). Moreover, the mutations did not disrupt the negative regulation of H-NS, because the activity level without LeuO induction was similar to the wild type. This shows that the LeuO (I) site is required for LeuO derepression. However, the mutations introduced on pRO310 bIV–mt2 derepressed partially the expression without LeuO induction; although there was still response to LeuO activation (Fig. 5B). These observations are consistent with the notion that these LeuO (II) site mutants map indeed at a site of high affinity for H-NS at box IV (Fig. 4A, C and D), resulting in partial derepression. The positive effect of LeuO on pRO310 bIV–mt2 is also consistent with an unaltered LeuO (I) binding site. Moreover, the mutated TATATATCCT region at bIV–mt2 corresponds to a highly protected region by H-NS (Fig. 4D).

Electrophoretic mobility shift assays were preformed to examine the effect of the mutations on LeuO and H-NS binding to the –226 to + 27 F2 fragment, using as negative control fragment F5 (–310 to –153) (Fig. 5C). As can be observed, F2 derived from pRO310 bIV–mt1 did not shift with LeuO; however, it bound H-NS in agreement to the genetic data where these changes only alter the derepressing effect of LeuO (Fig. 5B). F2 from pRO310 bIV–mt2 bound to LeuO and also to H-NS (Fig. 5C), but at higher H-NS concentrations as compared with F2 from the wild type and from pRO310 bIV–mt1 (Figs 4C and 5C).

Different *ompS1–lacZ* fusions carrying various lengths of the 5′ regulatory region were transformed either with pFM*Trc12* or with pFM*TrcleuO*-50. pRO310, pRO266 and pRO153 fusions responded to the LeuO-positive effect; however, the pRO117 fusion was not positively regulated by LeuO, being similar to the pRO310–d4 (Fig. 5D). This is also consistent with the notion of the relevance of the LeuO (I) binding site (at –134 to –125) (Fig. 4F).

The fact that a set of mutations (mt1) on the LeuO (I) binding site drastically affected LeuO but not H-NS activity, and that a second set (mt2) on LeuO (II) binding site partially affected H-NS activity but not the effect of LeuO (Fig. 5B), indicates that the zone around OmpR-binding box IV is key in the positive and negative regulation of *ompS1*.

LeuO acted as a derepressor, displacing H-NS from the ompS1 *regulatory region*

The highest level of *ompS1* expression was attained either upon induction of LeuO in the wild type or else in an *hns stpA* background (Figs 1–3). In order to assess the effect of LeuO in an *hns stpA* background, an *E. coli* double *hns stpA* mutant was used as the dominant negative *hns* system described earlier (Fig. 3) could not be used because of plasmid incompatibility between pFM*TrcleuO*-50 and pT6-HNS-G113D. We thus tested two *ompS1* fusions, pFV4 and pFV5, being equivalent to pRO88 and pRO310, respectively, but with the appropriate antibiotic resistance marker. The level of *ompS1* expression in pFV5 in such *hns stpA* background did not increase in the presence of LeuO (Fig. 6A), which is consistent with the notion that it is acting as a derepressor antagonizing H-NS and StpA action, and where LeuO is not acting by recruiting RNA polymerase to the *ompS1* promoter.

Competitive EMSAs between LeuO and H-NS for binding to the *ompS1* regulatory region were performed to examine the effect of LeuO on H-NS bound to DNA. We examined the single binding of H-NS and LeuO to the F1 fragment from the 5′ regulatory region (Fig. 4A; Table 1). Both proteins bound to F1-forming DNA–protein complexes (Fig. 6B). F1 was first incubated with H-NS and then increasing amounts of LeuO were added. The H-NS–DNA complexes, the displaced H-NS protein and the LeuO–DNA complexes were detected by immunoblot with anti-*Myc* antibodies for H-NS and with a polyclonal antibody to LeuO respectively (Fig. 6B). As can be observed,

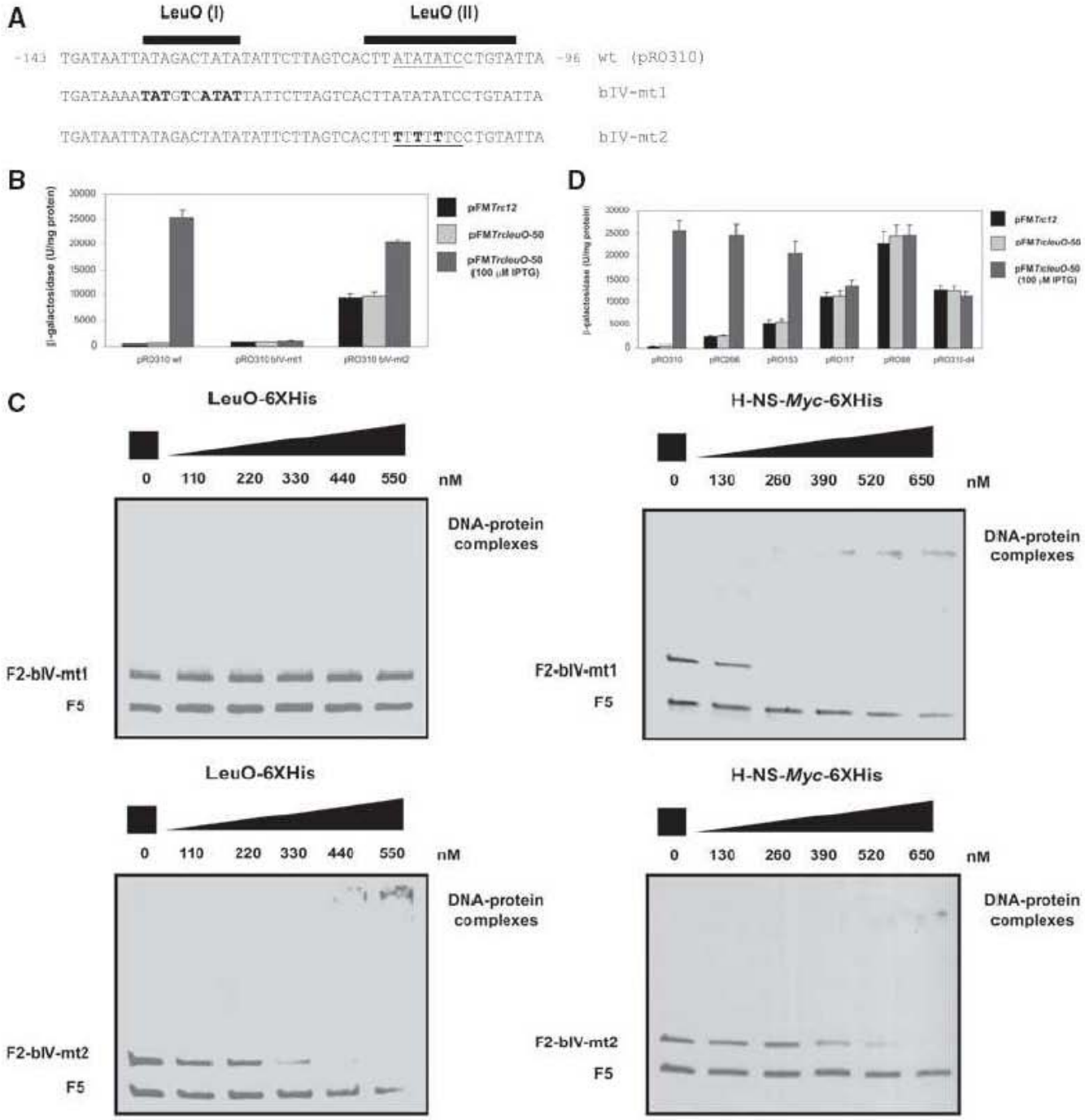

Fig. 5. Relevance of the LeuO (I) binding site.
A. Mutagenesis of *ompS1* LeuO binding sites I and II: nucleotide sequence of the wild type (wt) and of the mt1 and mt2 box IV mutated sites. The underlined sequence presents homology to the H-NS nucleation site of the *proU* gene (AATATATCGA) (Bouffartigues *et al*., 2007); the corresponding sequence in mt2 is also underlined.
B. β-Galactosidase activity of the *ompS1–lacZ* wild type reporter fusion pRO310 and of the pRO310 bIV–mt1 and pRO310 bIV–mt2 derivatives in the presence of either plasmid vector pFM*Trc12* or of the cloned *leuO* gene in plasmid pFM*TrcleuO*-50 without and with induction (100 μM IPTG).
C. EMSA showing the binding of various concentrations of LeuO and H-NS to mutated (bIV–mt1, bIV–mt2) F2 fragments using F5 as negative control.
D. β-Galactosidase activity of *ompS1–lacZ* reporter fusions carrying either 310, 266, 153, 117 or 88 nucleotides upstream of the P1 transcriptional start point; and of the 310 construct deleted for box IV.

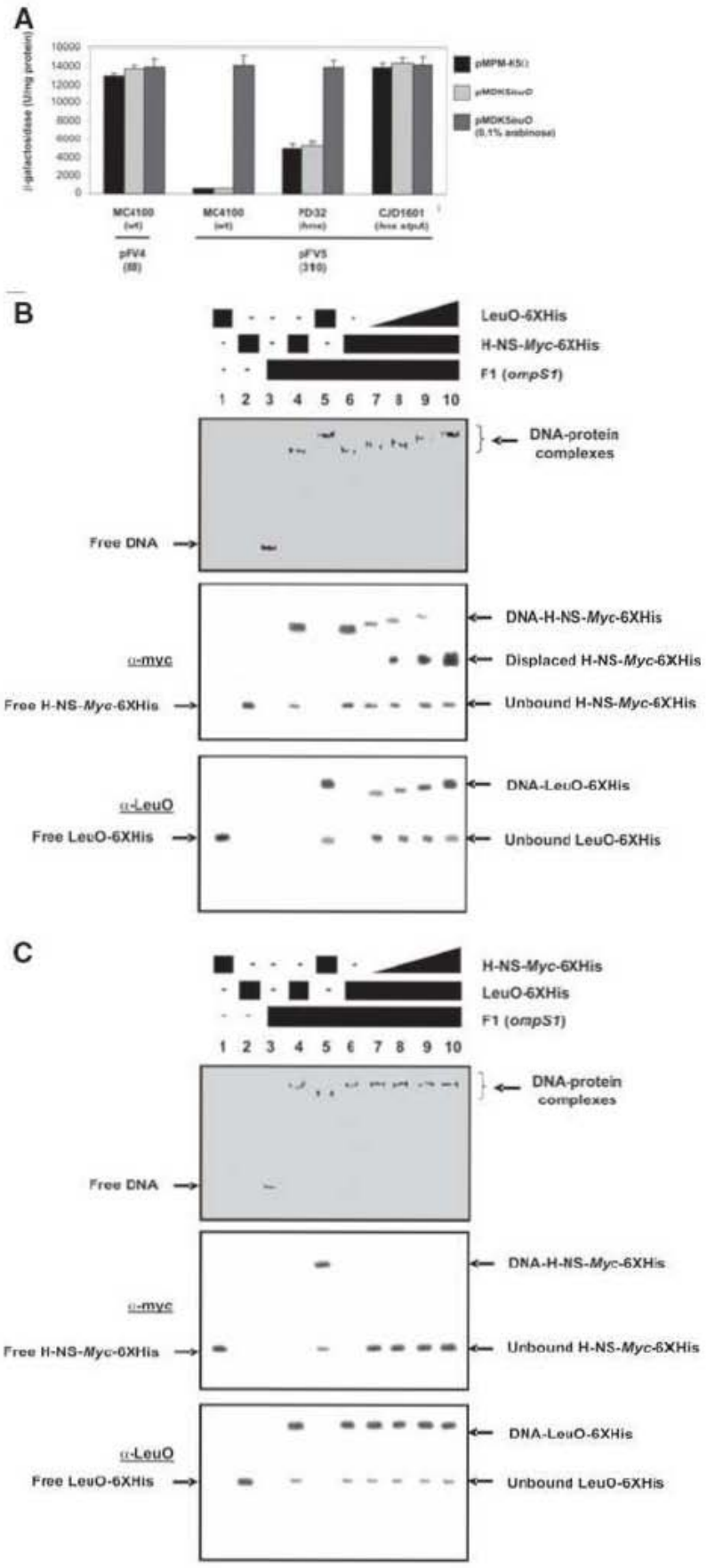

Fig. 6. LeuO as a derepressor for *ompS1* expression.
A. β-Galactosidase activity of the *ompS1–lacZ* reporter fusion pFV5 in *E. coli* wild type and mutants in *hns* and *hns stpA* in presence of pMPM-K5Ω, the pMDK5*leuO* with and without induction (0.1% arabinose) were evaluated. The pFV4 was included as a control in the wild type (wt) strain.
B. Competitive EMSA between H-NS, which was added first, and LeuO on the complete *ompS1* regulatory region F1 (100 ng in 20 µl) (lanes 3–10) (Fig. 5A). The upper panel shows the ethidium bromide-stained gel, the middle and lower panels show the immunoblot detection of the free H-NS or bound to DNA and of the free LeuO or bound to DNA respectively. H-NS was added at 0 nM (lanes 1, 3 and 5), 570 nM (lanes 2, 4 and 6–10), and LeuO was added at 0 nM (lanes 2, 3, 4 and 6), 165 nM (lane 7), 330 nM (lane 8), 500 nM (lane 9) and 665 nM (lanes 1, 5 and 10).
C. Competitive EMSA between LeuO, which was added first, and H-NS on F1. The upper panel shows the ethidium bromide-stained gel, the middle and lower panels show the immunoblot detection of the H-NS and of LeuO respectively. H-NS was added at 0 nM (lanes 2, 3, 4 and 6), 142 nM (lane 7), 285 nM (lane 8), 427 nM (lane 9), 570 nM (lanes 1, 5 and 10), and LeuO was added at 0 nM (lanes 1, 3 and 5), 665 nM (lanes 2, 4 and 6–10).

increasing amounts of LeuO modified the H-NS–DNA complexes causing a displacement of H-NS from the bound DNA. Moreover, this displaced H-NS migrated higher than the unbound H-NS, suggesting that the displaced protein might be in an oligomeric state. Similar experiments showed that StpA was displaced by LeuO (data not shown).

Competitive EMSAs were performed incubating first LeuO with F1 and adding subsequently H-NS (Fig. 6C). Interestingly, DNA-bound LeuO was not displaced by the subsequent addition of H-NS (Fig. 6C). This indicated that LeuO does effectively displace H-NS from the *ompS1* regulatory region and that, once bound, it prevents H-NS binding.

Discussion

LeuO, a regulator of the LysR family, was found to be a positive effector of the *ompS1* gene in *Salmonella*. Previously, it was reported that LeuO positively regulates *ompS2* (Fernández-Mora *et al.*, 2004). Furthermore, the regulation of these two quiescent genes is dependent on the level of induction of *leuO* (Fig. 1). Consequently, this could reflect a temporal expression in the cell dependent on the level of LeuO, where the expression of each porin was mutually exclusive. The fact that *ompS2* expression diminishes at the highest levels of *leuO* induction (Fig. 1A) could mean that LeuO can act as a repressor aside rather than as an activator, for some genes. The repression of *ompS2* observed at the higher levels of induction of the *leuO* gene could be due to the competition of LeuO for the binding site of the OmpR transcriptional activator as *ompS2* expression is dependent of LeuO and OmpR (Fernández-Mora *et al.*, 2004).

The maximum level of *ompS1* expression was reached either by the action of LeuO or in an *hns stpA* background (Figs 1, 2, 3 and 6A). StpA has been described as a molecular back-up that acts in the absence of H-NS, having both overlapping and distinct functions (Sondén and Uhlin, 1996; Zhang *et al.*, 1996). In uropathogenic *E. coli* the gene expression pattern of an *hns stpA* double mutant differed from that of the *hns* single mutant, suggesting the existence of a second regulon that is dependent on the presence of both regulators, presumably in the form of a heteromeric complex (Müller *et al.*, 2006). Thus, the regulatory model for *ompS1* presented also involves StpA as a negative regulator. The negative effect of StpA on *ompS1* was only observed in the *hns* background; in the *stpA* background *ompS1* expression was as in the wild type (Fig. 3). Such dual control would probably play a role in the silencing of genes acquired by horizontal transfer, which would be required in an environment other than the standard laboratory conditions (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). Interestingly, coding sequences for both H-NS and StpA are present in several enterobacteriaceae including *E. coli* and *S. enterica*, members of the *Shigella* genus and in *Erwinia carotovora* (Madrid *et al.*, 2007). Moreover, StpA as H-NS can interact with other regulatory proteins such as the Hha/YmoA family (Paytubi *et al.*, 2004). StpA also can function as an RNA chaperone as in the case of the *ompF* porin gene, which positively regulates at the post-transcriptional level by means of stabilizing MicF, an antisense RNA of the *ompF* transcript (Deighan *et al.*, 2000).

The P1 and P2 *ompS1* promoters are regulated in a hierarchical manner. That is, the OmpR response regulator differentially determines transcription from P1 hindering transcription from P2, which is active in the absence of OmpR (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). Such hierarchy was maintained upon derepression by LeuO (Fig. 2B). Thus, whereas the induction of *ompS2* expression by LeuO is from a sole OmpR-dependent promoter (Fernández-Mora *et al.*, 2004), the induction of *ompS1* can occur independent of OmpR.

The data show that in *S.* Typhi LeuO caused derepression of the pRO310 fusion, reaching the maximum levels as observed from the pRO88 fusion, both in the wild type and in the *hns* background (Fig. 2A). As in the *hns stpA* background such maximum level was also observed (Figs 3 and 6A), we postulate that LeuO acts as a derepressor and not as an activator in *ompS1* regulation (Fig. 6A). This highest level of expression allowed the detection of OmpS1 as a major OMP (Figs 1B, 2C and 3B). Together with this genetic evidence, it was found that LeuO could displace H-NS from the 5′ regulatory region (Fig. 6B and C); and a binding site for H-NS, StpA and LeuO was mapped around the previously defined OmpR-binding box IV, –124 to –107 bp upstream of the transcriptional start for promoter P1 (Fig. 4). The concurrence in the binding of all four regulators denotes a central key regulatory site that reflects a complex regulatory circuit (Fig. 4). Furthermore, it has been observed previously that the deletion of OmpR box IV, in the pRO310–d4 derivative, results in a considerable derepression of the pRO310 template both in the wild type and in the *hns* background, and in the abolishment of negative regulation in the *hns* mutant at high osmolarity (Fig. 5D, Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). These earlier observations fully concur with the model proposed here.

In terms of the DNA binding site for LeuO, it has not been extensively characterized. Nevertheless, the LeuO binding site (between –137 and –99) on *ompS1* shares the sequence ANTCA (Fig. 4F) with the binding sites at the *ompS2* and *leuO* genes, and with a site for LeuO activity on *dsrA* (Repoila and Gottesman, 2001; Chen *et al.*, 2003; Fernández-Mora *et al.*, 2004). Binding of LeuO resulted in what appears to be a DNA fold, which is revealed by the appearance of a DNase hypersensi-

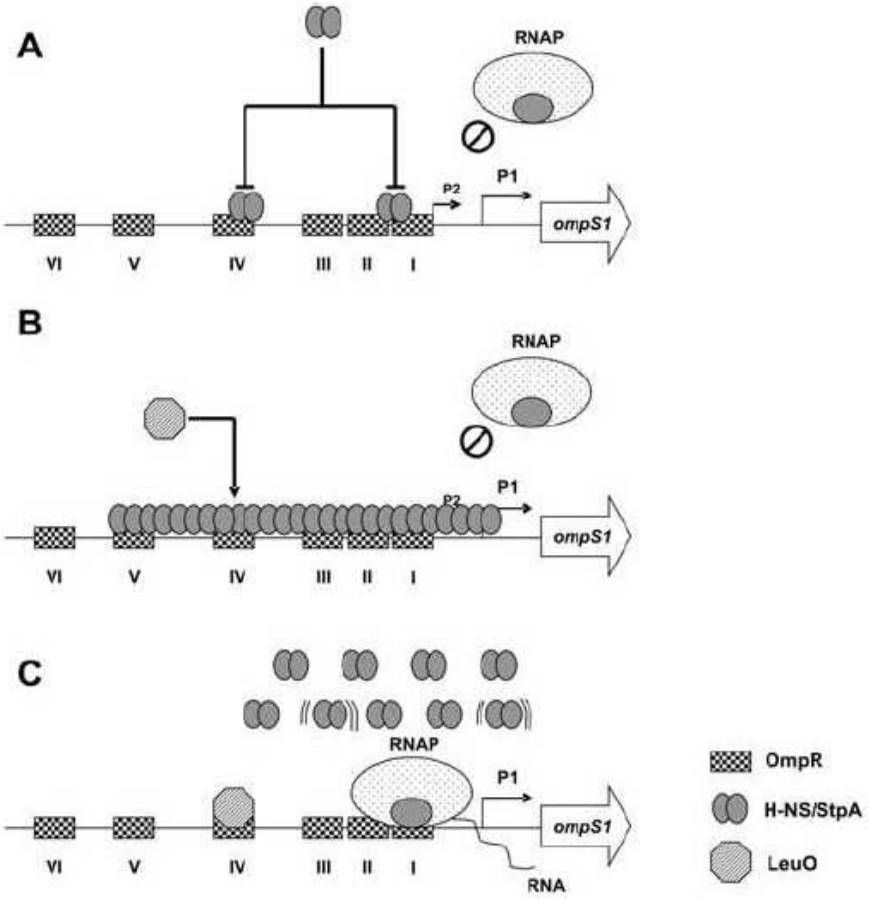

Fig. 7. Current model for the expression of the *S. enterica ompS1* gene. The P1 and P2 promoters are shown together with the six OmpR-binding boxes in the *ompS1* 5′ regulatory region.
A. Binding of H-NS to two nucleation sites at OmpR-binding boxes I and IV.
B. H-NS polymerization that impedes RNA polymerase activity.
C. Binding of LeuO to the LeuO (I) site resulting in the displacement of H-NS and derepression.

tive region as seen previously for *ompS2* (Fernández-Mora *et al.*, 2004). The introduction of punctual mutations (mt1) in the LeuO binding region showed that the LeuO (I) site at –134 to –125 was required for derepression of *ompS1* expression by LeuO and that it was independent of H-NS action (Fig. 5A and B). In addition, these mutations impeded LeuO binding (Fig. 5C). This idea was corroborated when different length gene reporter fusions that did not include the LeuO (I) site were not positively regulated by LeuO (Fig. 5E), even though one of them (pRO117) still included the LeuO (II) binding site. Hence, the mutated residues in mt1 appear to be determinant for LeuO binding as observed by EMSA (Fig. 5C). On the other hand, mutation (mt2) of the LeuO (II) binding site had a different effect (Fig. 5B). In the absence of LeuO, *ompS1* activity was partially derepressed, indicating that an H-NS binding site was affected as corroborated by EMSA and DNase footprinting (Fig. 5C and D). Most importantly, in mt2, LeuO binding and activity were preserved (Fig. 5B and C). Therefore, LeuO (I) appeared to be the key site for LeuO-mediated derepression. However, the LeuO (II) site could be required for enhancing the LeuO derepressor effect as it is protected by LeuO (Fig. 4F). Similarly, box 2 of the of *icsB* gene in *Shigella flexneri* has been shown to be critical for VirB-mediated derepression, in contrast to box 1 (Turner and Dorman, 2007).

H-NS and StpA bound at a downstream sequence located at –59 to –25 (Fig. 4A–C). Hence, this binding site and the one upstream at –164 to –114 might be nucleation sites for oligomerization of H-NS or StpA, as described for the *proU* gene (Bouffartigues *et al.*, 2007). This would lead to the formation of a putative repressor complex that blocks RNA polymerase (Figs 4A and 7). The region of –113 to –102, at the LeuO (II) binding site, forms part of an H-NS nucleation site as mutations in this site (mt2) resulted in the derepression of *ompS1* expression (Fig. 5A and B) and the DNase footprinting showed that this site had highest affinity for H-NS (Fig. 4D). Interestingly, the sequence at the LeuO (II) binding site (TATATATCCT) presents homology to the H-NS nucleation site of the *proU* gene (AATATATCGA) (Bouffartigues *et al.*, 2007) and the mt2 mutant at this site (TTTTTTTCCT) showed less affinity for H-NS (Fig. 5A and C). Another bincing site for H-NS was mapped downstream around OmpR box I (Fig. 4). Interestingly, it contains the motif TAAATATCTT at –62 to –53 between OmpR-binding boxes II and I. These observations support our model that involves two sites of interaction of H-NS necessary for the repression as was seen for the *proU*

gene (Bouffartigues *et al.*, 2007), probably forming a nucleoprotein complex. However, fragment F4 (–114 to +27) that carries both putative nucleation sites did not shift with H-NS (Fig. 4C). Nevertheless, fragments F3 (–164 to +27) and F6 (–153 to +1) that contain both proposed nucleation sites plus upstream and downstream sequences do shift with H-NS (Fig. 4C), indicating a possible role of such adjacent regions in the enhancement of DNA–H-NS complexes as was seen in the DNase footprinting (Fig. 4D). In this respect, the pRO310–d4 fusion, deleted for –135 to –105, did not reach the maximum expression level represented by pRO88 (Oropeza *et al.*, 1999), an observation similar to that for the *proU* gene where the mutation of both nucleation sites derepressed the expression of the gene but not to its maximum level (Bouffartigues *et al.*, 2007). The fragment derived from pRO310–d4 diminished its affinity to H-NS with respect to the wild type but still shifted (Flores-Valdez *et al.*, 2003). This further indicates that probable existence of such other sites of H-NS interaction upstream and downstream of the deleted region. In accordance, it has been proposed that for the regulation of the *proU* gene the H-NS binding region consists of a series of small binding sites forming modules that are organized around two nucleation sites (Bouffartigues *et al.*, 2007).

Hence, our model for *ompS1* regulation involves the LeuO, OmpR, H-NS and StpA regulators (Fig. 7). We propose that LeuO binds at –134 to –125, at LeuO (I) box, displacing H-NS and StpA and modifying the DNA local architecture, while OmpR would bind in the proximity of the promoter region differentially regulating P1 or P2 (Fig. 7). The interaction of H-NS and StpA solely with a second binding site (–59 to –25) would not be enough to form the nucleorepressor complex, as its stability would be dependent on the binding of H-NS and StpA to both sites. Moreover, previous genetic data show that the removal of upstream regions from –310 to –88 gradually caused the derepression of *ompS1* expression (Fig. 5C, Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003) which, together with the H-NS DNase footprinting data (Fig. 4D), suggest that H-NS binds at the two nucleation sites proposed and polymerizes throughout the entire 5′ upstream region, a type of interaction that has been proposed for H-NS (Dorman, 2007). In addition, no significant intrinsic curvature was found *in silico* between the two putative nucleation sites, which is not consistent with the formation of a repressive loop.

For the *leuO* locus, LeuO-mediated regulation involves the binding of the protein to the promoter region blocking the progress of the nucleorepressor filament (Chen and Wu, 2005). Our model is different by various criteria. LeuO acts as a derepressor for *ompS1* expression by binding upstream of the promoter and not in the proximal region. In addition, our model contemplates that LeuO physically does not block the progress of H-NS but rather causes a displacement of H-NS, hence preventing its binding at the nucleation sites and its polymerization, and causing a change in DNA structure as shown by the formation of a DNase hypersensitive site (Figs 6B and C and 4F). Changes in the DNA conformation caused by a derepressor were seen for the regulation of *icsB* gene by VirB (Turner and Dorman, 2007) and for the *gadA* and *gadBC* genes by means of GadX and GadW in *E. coli* (Tramonti *et al.*, 2006).

Thus, members of several families of transcriptional regulators have been described as antagonists of H-NS: VirF (AraC/XylS family) (Tobe *et al.*, 1993), RovA (SlyA/Hor) (Heroven *et al.*, 2004) and Ler (H-NS/StpA) (Bustamante *et al.*, 2001), among others. The studies reported here involve a regulator of the LysR family, LeuO, as a derepressor by displacement of H-NS and StpA.

Although there are only a few known genes directly regulated by LeuO, the new mechanism described here illustrates the versatility in LeuO function. In this respect, for instance, *Yersinia enterocolitica* RovA acts as a derepressor of H-NS, differentially binds to two genes: *inv* and *rovA* (Heroven *et al.*, 2004). Similarly, differential binding were observed for the GadW and GadX regulators in the regulation of *gadA* and *gadBC* in *E. coli* (Tramonti *et al.*, 2006).

The observation that both *ompS1* and *ompS2* were regulated by LeuO at different levels of induction opens the possibility that there is a regulon in *S. enterica*, where various genes are expressed at different levels according to the concentration of LeuO in the bacterial cell.

Experimental procedures

Bacterial strains, plasmids and recombinant DNA techniques

Bacterial strains and plasmids used in this study are listed in Table 2. DNA manipulations were performed according to standard protocols (Sambrook and Russell, 2001). Oligonucleotides used for amplification by PCR were provided by the Oligonucleotide Synthesis Facility at our institute and are listed in Table 1. PCRs were performed with *Taq* DNA polymerase (Invitrogen), according to the instructions by the manufacturer. The one-step mutagenesis procedure described by Datsenko and Wanner (2000) for bacterial chromosomal genes was used to generate gene deletions and replacements for antibiotic resistance markers.

Generation of point mutants in the LeuO binding site

The mutations introduced were performed using the strategy of inverse PCR. We used as template the pRO310 fusion, designing complementary primers that carry the desired changes. The primers used were: 5′-AAATGATAAAATA

Table 2. Bacterial strains and plasmids.

Strains o plasmid	Genotype y/o relevant markers	Reference
E. coli		
MC4100	F′ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsH*	Casadaban *et al.* (1976)
PD32	MC4100 *hns*-206::Ap^r	Dersch *et al.* (1993)
JPMC1	MC4100 Δ*hns*::Km^r	Barba *et al.* (2006)
CJD1600	MC4100 Δ*stpA*::Tc^r	Deighan *et al.* (2000)
CJD1601	CJD1600 Δ*stpA*::Tc^r*hns*-206::Ap^r	Deighan *et al.* (2000)
BL21(DE3)	F$^-$*ompT gal* (*dcm*) (*lon*) *hsdS*$_B$(r_B^- m_B^-) *met*(DE3)	Novagen
S. enterica		
IMSS-1	*Salmonella enterica* serovar Typhi 9.12, d, Vi serotype; Mexican reference clinical strain	Puente *et al.* (1987)
IMSS-41	IMSS-1 Δ*ompR*::Cm^r	Flores-Valdez *et al.* (2003)
STY*hns*99	IMSS-1 *hns*99::Km^r*mariner*	Flores-Valdez *et al.* (2003)
STY9941	STY *hns*99 Δ*ompR*::Cm^r	Flores-Valdez *et al.* (2003)
STY Δ*stpA*	IMSS-1 Δ*stpA*:: Cm^r	This study
STYS1163	IMSS-1 Δ*ompS1*::Km^r	This study
STYS241	IMSS-1 Δ*ompS2*::Km^r	This study
IMSSTN103	IMSS-1 derivative containing a *Tn*10::Cm insertion 160 bp upstream of the first ATG and 60 bp downstream of the promoter for the *leuO* gene	Fernández-Mora *et al.* (2004)
Plasmids		
pRO310	pMC1871-derived plasmid, containing a translational fusion of the *ompS1* 5′ regulatory region, up to 310–27 bp upstream of the transcriptional start point, to the *lacZ* reporter gene	Oropeza *et al.* (1999)
pRO88	pMC1871-derived plasmid, containing a translational fusion of the *ompS1* 5′ regulatory region, up to 88–27 bp upstream of the transcriptional start point, to the *lacZ* reporter gene	Oropeza *et al.* (1999)
pFV5	pACYC184-derived plasmid, carrying the SalI fragment of the pR0310 fusion	This study
pFV4	pACYC184-derived plasmid, carrying the SalI fragment of the pR088 fusion	This study
pKFV310	pKK232-8-derived plasmid, containing a transcriptional fusion of the *ompS1* 5′ regulatory region, up to 310–27 bp upstream of the transcriptional start point, to the *cat* reporter gene	This study
pKFV88	pKK232-8-derived plasmid, containing a transcriptional fusion of the *ompS1* 5′ regulatory region, up to 88–27 bp upstream of the transcriptional start point, to the *cat* reporter gene	This study
pFM413	pMC1871-derived plasmid containing translational fusions of the *ompS2* 5′ regulatory region, up to 413–34 bp upstream of the transcriptional start point, to the *lacZ* reporter gene	Fernández-Mora *et al.* (2004)
pT6-HNS-G113D	pMPM-T6Ω encoding an *hns* mutant allele (G113D) (negative dominant); p15A1	Bustamante *et al.* (unpubl. data)
pFM*Trc12*	p*Trc*99A modified with replication origin p15A1; Ap^r	Fernández-Mora *et al.* (2004)
pFM*TrcleuO*-50	pFM*Trc12* carrying the *S.* Typhi *leuO* gene fused to His$_6$ under *tac* promoter	This study
pMDK5*leuO*	pMPM-K5Ω-derived plasmid, carrying the NcoI-BamHI fragment of pFM*TrcleuO*-50	This study
pMDH-NS	pBAD-*Myc*-His$_6$ C carrying the *S.* Typhi *hns* gene fused to *Myc*-His$_6$ under the arabinose promoter	This study
pMDStpA	pET-28b(+) carrying the *S.* Typhi *stpA* gene fused to *T7*-His$_6$ under *T7* promoter	This study
pFC63	pBR325 carrying the *S.* Typhi *ompS1* under its own promoter	Fernández-Mora *et al.* (1995)
pKD46	Red recombinase system under the arabinose promoter; Ap^r	Datsenko and Wanner (2000)
pKD3	Template plasmid containing the Cm cassette for lambda Red recombination	Datsenko and Wanner (2000)
pKD4	Template plasmid containing the Km cassette for lambda Red recombination	Datsenko and Wanner (2000)

TGTCATATTCTTAG-3′ and 5′-CTAAGAATAATATGACATAT TTTATCATTT-3′ (pRO310 bIV–mt1); 5′-CTTAGTCACTTT TTTTTCCTGTATTATAAA-3′ and 5′-TTTATAATACAGGAAAA AAAAGTGACTAAG-3′ (pRO310 bIV–mt2). The PCR products were digested with DpnI to eliminate the template and transformed in MC4100. The candidates were sequenced to verify their identity.

Bacterial culture, β-galactosidase and CAT assays

Bacteria were grown in nutrient broth (low osmolarity) or nutrient broth plus 0.3 M NaCl (high osmolarity) at 37°C and collected at mid-logarithmic phase. The culture conditions and microplate protein and β-galactosidase assays were as previously described (Flores-Valdez *et al.*, 2003). CAT assays and protein quantification to calculate Cloramphenicol acetyl transferase (CAT)-specific activities were performed as described previously (Martinez-Laguna *et al.*, 1999).

OMP purification and electrophoresis

Outer membrane proteins were prepared as a Triton X-100 insoluble fraction and scaled down to a mini-prep level as described previously (Fernández-Mora *et al.*, 2004). They were separated by SDS-12% polyacrylamide gel electrophoresis (PAGE) and visualized by staining with Coomassie brilliant blue.

Primer extension analysis

Total RNAs were isolated from samples of cultures grown in nutrient broth at 37°C and collected at mid-logarithmic phase. Five micrograms of total RNA (for *ompA*) or 25 μg of total RNA (for *ompS1*), isolated by using commercial kit (Rneasy, Qiagen), was denatured at 90°C for 3 min and then slowly cooled to 50°C. The RNA was annealed with [γ-^{32}P]ATP-labelled *ompA*-PE 5′-TTTGCGCCTCGTTATCATCCAA-3′

(annealing from position +3 to –24 with respect to the translational start site) or primer *ompS1*-PE 5′-TTGCT GCGCCTGCCACTAATAAC-3′ (annealing from +57 to +35 with respect to the translational start site). Primers were extended with Moloney murine leukaemia virus reverse transcriptase at 37°C for 2 h, and the extended products were collected with a Microcon-30 microconcentrator (Amicon) and analysed by electrophoresis in urea–8% polyacrylamide gels alongside sequencing ladders.

*Isolation and purification of H-NS-*Myc*-6XHis, StpA-T7-6XHis and LeuO-6XHis proteins*

The *S.* Typhi *hns* structural gene was amplified by PCR using with the forward primer 5′-ACTACCATGGGCGAAGCAC TTAAAATTC-3′ and the reverse primer 5′-GTACTCGA GTCCTTGATCAGGAAATCTTCC-3′. Then digested with NcoI-XhoI and finally cloned into pBAD/*Myc*-His C. The *S.* Typhi *stpA* structural gene was amplified with the forward primer 5′-ATTGCCATGGATTTGATGTTACAGAACTTAAAT-3′ and the reverse primer 5′-TTATCCGCATATGGATTAA GAAATCATCCAGAGATT-3′, digested with NcoI-NdeI and cloned into pET-28b(+). LeuO was previously cloned to the expression vector p*Trc*99A (Fernández-Mora *et al.*, 2004). Purification of H-NS-*Myc*-6XHis, StpA-*T7*-6XHis and LeuO-6XHis proteins was performed with Ni-nitrilotriacetic acid resin (QIAExpress, Qiagen) according to the instructions of the manufacturer and the modifications established by Barba *et al.* (2006). Briefly, *E. coli* BL21(DE3) carrying the pMDHNS, pMDStpA or pFM*TrcleuO*-50 plasmids (Table 2) was grown to mid-logarithmic phase. L (+)-arabinose (Sigma-Aldrich) was added to a final concentration of 0.1%, or IPTG to 1 mM, and the bacteria were incubated to 200 r.p.m. for 4 h at 37°C. Cells were then pelleted by centrifugation, resuspended in urea buffer [8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl (pH 8.0)] and disrupted by sonication. The suspension was centrifuged, and the supernatant was filtered through a Ni-nitrilotriacetic acid agarose column (QIAExpress, Qiagen); the column was washed with urea buffer at pH 8.0 and urea buffer at pH 6.0, and finally the bound protein was eluted with urea buffer at pH 4.5. Fractions containing purified H-NS-*Myc*-His_6, StpA-*T7*-His_6 and LeuO-His_6 proteins were selected after SDS-PAGE. The selected fractions were loaded into a Slyde-A-Lyzer 10K cassette (Pierce) and gradually dialysed at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20% glycerol, 0.5 M NaCl, 0.1% Triton X-100, various amounts of urea (4, 1 and 0.2 M), which was changed every hour. The final dialysis was done in storage buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20% glycerol, 240 mM NaCl, 0.1% Triton X-100 and 3 mM EDTA, and aliquots of the purified proteins were stored at –70°C. Protein concentrations were determined by the Bradford procedure.

Electrophoretic mobility shift assays

Polymerase chain reactions that generated various *ompS1* products encompassing the 5′ regulatory region were obtained, using plasmid pRO310 as template and series of oligonucleotide primers (Table 1). The fragments (~100 ng in 20 µl) were mixed with increasing concentrations of H-NS-*Myc*-6XHis or StpA-*T7*-6XHis, in the presence of the binding buffer H/S 10× (400 mM HEPES, 80 mM $MgCl_2$, 500 mM KCl, 10 mM DTT, 0.5% NP40 and 1 mg ml^{-1} BSA). They were incubated for 20 min at room temperature and then separated by electrophoresis in 6% native polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. For LeuO-6XHis the mixture was in the presence of binding buffer L 10× (20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA and 20% glycerol). LeuO was incubated with DNA for 30 min at room temperature. The DNA bands were visualized by staining with ethidium bromide. Competitive EMSAs were performed incubating first with one protein and then incubating with DNA; followed by the addition of the other protein and incubation.

DNase I footprinting assays

Plasmid pRO310 was used as a template for PCR amplification of the *ompS1* 5′ regulatory region. A ^{32}P-labelled oligonucleotide complementary to the 5′-coding sequence of *ompS1* (5′-TACTTTTCTTTTCATTTTTT-3′) and an oligonucleotide complementary to –253 to –228 bp relative to the transcriptional start P1 (5′-TCGCTCAAGAAATTAAATT AAAAAA-3′) were used. The binding of LeuO to the labelled DNA fragment was performed at room temperature; in a buffer that contained 20 mM HEPES (pH 7.9), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol and 1 µg of poly(dI-dC). The reaction mixture was treated with different concentrations of DNase I. The DNase footprinting with H-NS was performed at 6°C. The DNA segments were separated by electrophoresis in 8% polyacrylamide–8 M urea gels alongside sequencing ladders.

Western blotting

Samples subjected to native-PAGE (6% polyacrylamide), and transferred to 0.22-µm-pore-size nitrocellulose membranes (Amersham, UK) in a semidry electrophoresis unit (Bio-Rad). Membranes were blocked with 5% non-fat milk and incubated with anti-*Myc* or anti-*T7* monoclonal antibodies (Sigma), or anti-LeuO polyclonal antibodies. Membranes were washed with (PBS 1×)-0.05% Tween 20, immunostained with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Pierce), for monoclonal or polyclonal first antibodies respectively, and developed with Chemiglow chemiluminescence reagents according to the manufacturer (Alpha Innotech).

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6.2 La curvatura estática del DNA reprime la expresión de *ompS1*

La curvatura estática del DNA tiene un papel relevante como elemento de regulación genética en la transcripción bacteriana. Previamente, habíamos encontrado que la región reguladora de *ompS1* presentaba zonas con curvaturas intrínsecas hacia el 5' (Flores-Valdez *et al*., 1995). Usando el software MUTACURVE, se predijeron mutaciones que disminuían y restablecían la curvatura del DNA hacia el 5'. La curvatura fue disminuida por mutagénesis sitio dirigida de solamente dos nucleótidos y restablecida en los mismos dos nucleótidos pero con mutaciones diferentes. La disminución de la curvatura intrínseca incrementó la expresión de *ompS1* y en la disminución de la afinidad de las proteínas silenciadoras H-NS y StpA hacia la región reguladora de *ompS1*. Estas mutaciones estuvieron en una región que no contenía el sitio de nucleación de H-NS y es consistente con que el efecto sobre la expresión fue debido a cambios en la topología del DNA.

The DNA static curvature has a role in the regulation of the *ompS1* porin gene in *Salmonella enterica* serovar Typhi

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The DNA static curvature has been described to play a key role as a regulatory element in the transcription process of several bacterial genes. Here, the role of DNA curvature in the expression of the *ompS1* porin gene in *Salmonella enterica* serovar Typhi is described. The web server MUTACURVE was used to predict mutations that diminished or restored the extent of DNA curvature in the 5′ regulatory region of *ompS1*. Using these predictions, curvature was diminished by site-directed mutagenesis of only two residues, and curvature was restored by further mutagenesis of the same two residues. Lowering the extent of DNA curvature resulted in an increase in *ompS1* expression and in the diminution of the affinity of the silencer proteins H-NS and StpA for the *ompS1* 5′ regulatory region. These mutations were in a region shown not to contain the H-NS nucleation site, consistent with the notion that the effect on expression was due to changes in DNA structural topology.

INTRODUCTION

Salmonella enterica serovar Typhi (*S.* Typhi) is the aetiological agent of typhoid fever in humans (Pang *et al.*, 1998). In our laboratory we have identified the *S.* Typhi *ompS1* gene that encodes the OmpS1 quiescent porin belonging to the OmpC/OmpF superfamily (Fernández-Mora *et al.*, 1995). OmpS1 has been reported to have a role in swarming motility, biofilm formation and virulence in mice (Toguchi *et al.*, 2000; Mireles *et al.*, 2001; Rodríguez-Morales *et al.*, 2006).

Expression of *ompS1* is dependent on two overlapping promoters, P1 and P2. The P1 promoter is dependent on the OmpR response regulator. The P2 promoter does not require OmpR for activation, being active only in the absence of OmpR (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003; De la Cruz *et al.*, 2007). Another key element in the transcriptional regulation of *ompS1* is the global regulatory protein H-NS, a nucleoid protein of 137 amino acids (15 kDa) that negatively regulates its expression (Flores-Valdez *et al.*, 2003; De la Cruz *et al.*, 2007). In *Salmonella*, H-NS has been proposed to selectively silence horizontally acquired genes by targeting sequences with a GC content lower than the resident genome, regulating around 12 % of its genes (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). StpA, an H-NS paralogue, was found to repress *ompS1* in an *hns* background; and LeuO, a LysR-type regulator, positively regulates *ompS1* expression by antagonizing H-NS and StpA (De la Cruz *et al.*, 2007). LeuO has been implicated in several functions, such as stress resistance, virulence and biofilm formation (VanBogelen *et al.*, 1996; Fang *et al.*, 2000; Majumder *et al.*, 2001; Tenor *et al.*, 2004; Lawley *et al.*, 2006; Moorthy & Watnick, 2005; Rodríguez-Morales *et al.*, 2006). Recently, our group has described several genes regulated by LeuO in *S.* Typhi (Hernández-Lucas *et al.*, 2008).

In bacterial genomes, the recognition of their binding targets by regulatory proteins is commonly considered to be sequence-dependent, although DNA curvature plays a well-characterized role in many transcriptional regulation mechanisms in prokaryotes (Jáuregui *et al.*, 2003; Olivares-Zavaleta *et al.*, 2006). For example, static DNA curvature has been shown to activate transcription, facilitating the binding of RNA polymerase to promoters, or favouring the interaction of activator proteins (Pérez-Martín *et al.*, 1994; Gourse *et al.*, 2000). Curved DNA regions have also been found to repress transcription initiation, where DNA curvature generally plays an indirect role, being the target for the binding of specific silencer proteins or by stabilizing or enhancing a preexisting DNA loop, thus effectively blocking transcription of downstream regions (Olivares-Zavaleta *et al.*, 2006). In particular, such would be the case for the *ompF* porin gene (Mizuno, 1987).

Previously (Jáuregui *et al.*, 2003; Olivares-Zavaleta *et al.*, 2006), we performed computer analyses to study the prevalence of DNA static curvature in the regulatory

Abbreviation: EMSA, electrophoretic mobility shift assay.

regions of *Escherichia coli* and established that most of the global transcription factors (ArcA, CRP, FIS, FNR, Lrp, IHF and H-NS), as well as some specific regulators, have a tendency to regulate operons with curved DNA sequences in their upstream regions.

Here we present a topological analysis of the *ompS1* 5′ upstream regulatory region and the identification of a static curvature that plays an important role in the binding of H-NS and StpA, the silencer proteins of *ompS1*.

METHODS

Computational design of site-directed mutagenesis. In order to modify the extent of DNA static curvature in the regulatory region of *ompS1*, we used our web server MUTACURVE (http://www.ibt.unam.mx/biocomputo/dna_curvature.html). This server first evaluates the amplitude of the intrinsic DNA curvature of every nucleotide in a given sequence using the algorithm of Goodsell & Dickerson (1994), with the contribution matrices for rotational and spatial displacements reported by Satchwell *et al.* (1986). Secondly, centred at the maximum curvature value, the server evaluates the effect of every double point mutation in a window of 31 nucleotides (three helix turns) to select those changes that would produce a significant reduction in the intrinsic DNA curvature of the fragment. Finally, the server generates the curvature profiles of the original and mutated sequences for their comparative analysis.

Site-directed mutagenesis. Site-directed mutageneses were performed using complementary oligonucleotides that contained the mutations predicted by MUTACURVE (Table 2). The plasmid pRO310-wt was used as template to generate pRO310-mt and pRO310-re by inverse PCR (Table 1). The expected mutations were verified by nucleotide sequencing.

Analysis of static curvature. We amplified several DNA fragments using a series of oligonucleotides that encompass the *ompS1* regulatory region (Table 2). The PCR fragments were separated by 6% PAGE at 4 °C (polyacrylamide gels in 0.5× Tris/borate/EDTA at 4 °C, 70 V, without buffer recirculation), a condition favouring slower migration of curved DNA sequences (Falconi *et al.*, 1993; Flores-Valdez *et al.*, 2003; Olivares-Zavaleta *et al.*, 2006). Gels were stained with ethidium bromide and photographed in an Alpha Imager system (Alpha Innotech).

Bacterial culture and β-galactosidase assays. Bacteria were grown in nutrient broth (low osmolarity) or nutrient broth plus 0.3 M NaCl (high osmolarity) at 37 °C or 30 °C and collected at the mid-exponential phase. The culture conditions and microplate protein and β-galactosidase assays were as previously described (Flores-Valdez *et al.*, 2003).

Electrophoretic mobility shift assays (EMSAs). DNA fragments generated by PCR (100 ng) were incubated with increasing concentrations of H-NS, StpA or LeuO for 20 min at 4 °C in 20 µl. Binding buffers for each protein were described previously (De la Cruz *et al.*, 2007). The DNA fragments were separated by electrophoresis in 6% native polyacrylamide gels in 0.5× Tris/borate/EDTA buffer at 4 °C. Double-stranded oligonucleotides (50 ng) were incubated as above and electrophoresis was in 8% native polyacrylamide gels at room temperature. Gels were stained with ethidium bromide and photographed in an Alpha Imager system (Alpha Innotech).

RESULTS

Diminished and restored *ompS1* DNA curvature

Previously, our group, working with three DNA fragments of the 5′ upstream regulatory region of the *S.* Typhi *ompS1* porin gene, described the presence of intrinsic DNA curvature peaking around −230 and −149 bp upstream of the transcriptional initiation site at promoter P1 (Flores-Valdez *et al.*, 2003). MUTACURVE (Olivares-Zavaleta *et al.*, 2006), a software developed to determine the topographic profile of a particular DNA region *in silico*, was used to further define regions with the highest degree of curvature; one of them was found between −151 and −135 bp upstream of the P1 transcriptional start point (Fig. 1A). This region was of interest for further research in this work, because it is located in the vicinity of the binding sites of two main transcription factors, LeuO and H-NS, which regulate *ompS1* expression (De la Cruz *et al.*, 2007). In addition, our previous studies showed that removal of the region from −310 to −153 had a modest effect on derepression as compared to removal of the region between −153 and −117 (Oropeza *et al.*, 1999), which includes the curved region studied here. The advantage of the software

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or relevant markers	Reference
E. coli		
MC4100	F′ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*	Casadaban (1976)
***S.* Typhi**		
IMSS-1	*S. enterica* serovar Typhi 9.12, d, Vi serotype; Mexican reference clinical strain	Puente *et al.* (1987)
STY*hns*99	IMSS-1 *hns*99::Km^r *mariner*	Flores-Valdez *et al.* (2003)
Plasmids		
pRO310-wt	pMC1871-derived plasmid, containing a translational fusion of the *ompS1* 5′ regulatory region, up to 310–27 bp upstream of the transcriptional start point, to the *lacZ* reporter gene	Oropeza *et al.* (1999)
pRO310-mt	pRO310-derived plasmid carrying mutations A151G and G135T, lowering curvature	This work
pRO310-re	pRO310-derived plasmid carrying mutations C151G and A135T, restoring curvature	This work
pFM*Trc12*	p*Trc*99A modified with replication origin p15A1; Ap^r	De la Cruz *et al.* (2007)
pFM*TrcleuO*-50	pFM*Trc12* carrying the *S.* Typhi *leuO* gene fused to His_6 under *tac* promoter	De la Cruz *et al.* (2007)

Table 2. Oligonucleotide primers used

Bold letters indicate the mutations introduced.

Primer	Sequence (5′–3′)	Alignment	Reference
310b-1	TAGCCTTTTATCATTTATTTTATC	−303 Fwd	De la Cruz *et al.* (2007)
310b-2	ATGAGTTATGTGTTTTGATTTGA	−153 Rev	De la Cruz *et al.* (2007)
310b-3	CAAAGCATCAAATACATATAAAAA	−226 Fwd	De la Cruz *et al.* (2007)
310b-4	TGTTTCTATTTGGTTTTTTATAATAC	−76 Rev	De la Cruz *et al.* (2007)
310b-7	5′ATTCTTAGTCACTTATATATCCTGTAT TAT	−114 Fwd	De la Cruz *et al.* (2007)
310b-8	AATATGTAGCCACTTCAACAAAAC	+27 Rev	De la Cruz *et al.* (2007)
310-mt-5′	CACATAACTCATTAATAAAAAATGATA ATGATAGACTATATATTC	−164 Fwd	This work
310-mt-3′	GAATATATAGTCTATCATTATCATTTTT TATTAATGAGTTATGTG	−164 Rev	This work
310-re5′	CACATAACTCATTCATAAAAAATGATA ATAATAGACTATATATTC	−164 Fwd	This work
310-re3′	GAATATATAGTGTATTATTATCATTTTT TATGAATGAGTTATGTG	−164 Rev	This work

used was that it allowed the evaluation of the effect on DNA curvature of every double point mutation that encompassed the region from −226 to −76 (fragment Fb-wt) (Fig. 1A, B).

The mutations that affected the DNA curvature *in silico* were introduced into the wild-type *ompS1* 5′ upstream regulatory region near the H-NS binding site as defined previously by EMSA and DNase footprinting analysis (De la Cruz *et al.*, 2007). First, a double mutation was introduced to diminish the extent of DNA curvature (pRO310-mt). We then used this mutated DNA as substrate for a second PCR-mediated mutagenesis to introduce the required changes, different from the wild-type sequence, which would restore the original extent of DNA curvature (pRO310-re) (Table 1). The effects of these mutations on DNA bending were evaluated by the PCR amplification of these regions (Fb-wt, Fb-mt and Fb-re) and their corresponding analysis by PAGE at 4 °C (Falconi *et al.*, 1993; Flores-Valdez *et al.*, 2003; Olivares-Zavaleta *et al.*, 2006). The mutations had the expected effect on the electrophoretic migration as predicted *in silico* (Fig. 1A, C): the Fb-mt fragment migrated faster, consistent with an abolished or diminished curvature, while the Fb-re fragment co-migrated with Fb-wt, suggesting a restored curvature.

DNA curvature is required for the negative regulation of *ompS1* expression

In order to evaluate the effect of DNA curvature on the expression of *ompS1*, the *ompS1-lacZ* reporter activity of the plasmids pRO310-wt, pRO310-mt and pRO310-re was evaluated in *S.* Typhi. Interestingly, the pRO310-mt fusion, carrying the *ompS1* regulatory region with diminished curvature, was derepressed fivefold relative to the wild-type fusion (pRO310-wt), whereas the positive control, pRO310-re, carrying two point mutations in the same position as the ones introduced into pRO310-mt but restoring the curvature of the *ompS1* regulatory region, rendered the same low expression level as the pRO310 wild-type (Figs 1 and 2A). Thus, point mutations located in precisely the same position in the *ompS1* 5′ upstream region produced different effects on expression, depending on whether they did or did not change the extent of DNA curvature.

We have reported previously that H-NS silences *ompS1* expression (Flores-Valdez *et al.*, 2003; De la Cruz *et al.*, 2007). Thus, the effect of the mutations affecting the curvature was evaluated in an *hns* background (STY*hns*99) (Fig. 2B). The pRO310-mt fusion increased *ompS1* expression 40% with respect to the wild-type (Fig. 2B). Hence, the removal of the intrinsic curvature at −151 to −135 also derepressed *ompS1* expression in the *hns* background, although in a smaller proportion than in the wild-type background, where a fivefold effect was observed (Fig. 2A). This smaller effect could be due to the lowering of the affinity of the binding of StpA in an *hns* background, as shown in Fig. 5D.

Osmoregulation and thermoregulation of *ompS1* is not affected by changes in DNA curvature

The expression of *ompS1* is not osmoregulated in a wild-type background but is negatively regulated at high osmolarity in an *hns* background (Oropeza *et al.*, 1999; Flores-Valdez *et al.*, 2003). Several groups have reported that DNA static curvature is involved in the thermoregulation and osmoregulation of certain genes (Ramani *et al.*, 1992; Kaji *et al.*, 2003; Prosseda *et al.*, 2004). The expression of the pRO310-mt fusion was therefore analysed in a wild-type and *hns* background at low and high osmolarity and at 37 °C and 30 °C (Fig. 3). As can be observed, the removal of the DNA curvature did not have a major effect on expression in these growth conditions: although a slight derepression was observed at high osmolarity in the *hns* background, no effect of temperature was seen (Fig. 3).

LeuO and DNA curvature at the *ompS1* regulatory region

LeuO is an antagonist of H-NS and of its paralogue protein StpA, allowing *ompS1* expression; furthermore, the expres-

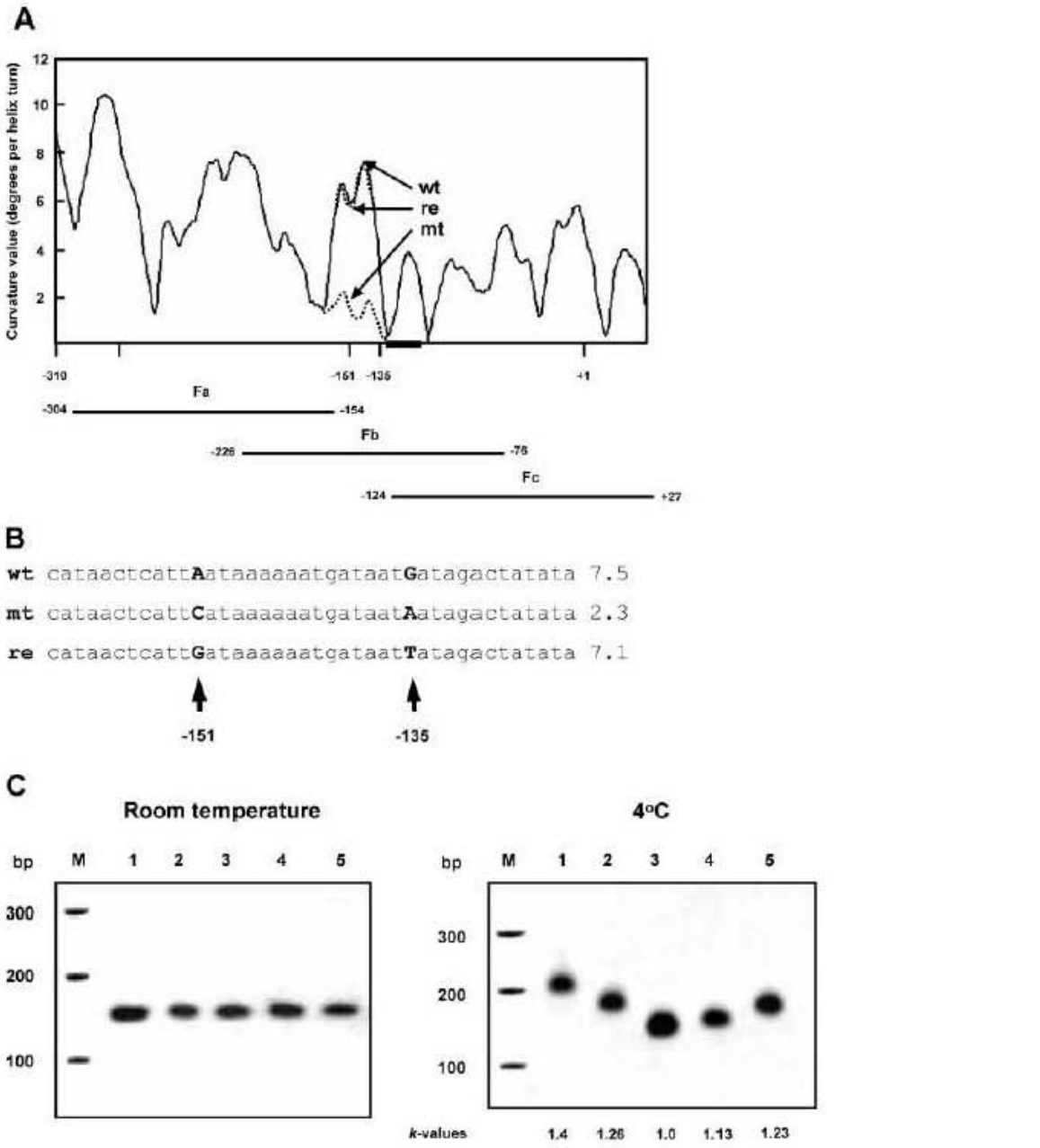

Fig. 1. *In silico* design of point mutations decreasing the DNA static curvature in the *ompS1* 5′ regulatory region and their effect on electrophoretic migration. (A) The DNA curvature value of each nucleotide at the *ompS1* 5′ upstream regulatory sequence was calculated and used to plot its curvature profile (solid line, wt). Centred at the nucleotide with the greatest curvature value, a window of 31 bp was used to look for the double nucleotide change that would produce the most significant reduction in intrinsic DNA curvature. This change was introduced into the original sequence and a new curvature profile was plotted (dotted line labelled mt). From these plots, one of the highest curvature regions was localized −151 to −135 bp upstream of the P1 transcription start site (indicated as +1). The thick bar on the *x*-axis represents the binding site for LeuO and H-NS, as detailed in Fig. 5A. Below the plot, the three DNA fragments (Fa, Fb and Fc) used for electrophoresis experiments are represented, with their coordinates. (B) Nucleotide sequences of the wild-type (wt), the mutant (mt) abolishing the curvature, and the mutant (re) restoring the curvature, at the −151 to −135 region, contained in the respective Fb fragments. The curvature values of the sequence are shown on the right and the relevant or mutagenized nucleotides are indicated in bold with their position upstream of the P1 transcriptional start site. (C) Electrophoretic migration of Fa, Fb and Fc wild-type fragments, and Fb-mt and Fb-re mutant fragments, at room temperature and at 4 °C. Lanes: M, 100 bp ladder DNA markers; 1, Fa-wt (−304 to −154); 2, Fb-wt (−226 to −76); 3, Fc-wt (−124 to +27); 4, Fb-mt (A151C, G135A); 5, Fb-re (C151G, A135T). The intrinsic bending of each fragment quantified by the *k*-value (Prosseda *et al.*, 2004) is shown at the bottom of the 4 °C gel.

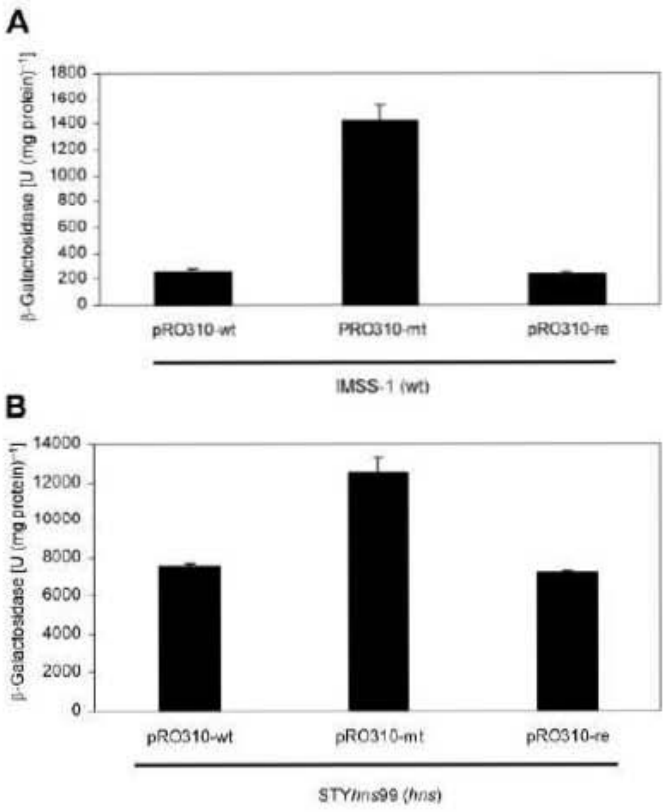

Fig. 2. *ompS1* expression upon elimination and restoration of 5′ intrinsic curvature at the Fb region: β-galactosidase activity of the *lacZ* fusions pRO310-wt, -mt and -re in *S.* Typhi wild-type (A) and STY*hns*99 (B).

sion of *ompS1* is dependent on the concentration of LeuO (De la Cruz *et al.*, 2007). The maximum level of *ompS1* expression was obtained when LeuO was induced at 100 μM IPTG from pFM*TrcleuO*-50, the same as that attained in a double *hns stpA* background (De la Cruz *et al.*, 2007). Hence, the effect of curvature on LeuO-mediated regulation of *ompS1* was examined by evaluating the expression of the pRO310-wt, pRO310-mt and pRO310-re fusions in the presence of cloned and expressed LeuO at different concentrations of IPTG (Fig. 4). Interestingly, the pRO310-mt fusion reached the maximum level when LeuO was induced at 50 μM IPTG, contrasting with pRO310-wt and pRO310-re, which reached the maximum level at 100 μM IPTG (Fig. 4). Furthermore, at 20 and 25 μM IPTG the expression was higher in the -mt construct than in the -wt and -re plasmids, at the same IPTG concentration. The observation that the degree of LeuO induction required to obtain the highest levels of *ompS1-lacZ* expression in strains carrying the plasmids with the curved *ompS1* regulatory region (plasmids pRO310-wt and pRO310-re) was almost double that required for those carrying the non-curved *ompS1* regulatory region (plasmid pRO310-mt) is consistent with the notion that diminution of the curvature enhanced the LeuO antagonistic effect on H-NS.

Effect of curvature on H-NS, StpA and LeuO binding

The effect of diminishing the DNA curvature of the *ompS1* regulatory region on the affinity of H-NS, StpA and LeuO was explored by EMSA. The binding of these proteins was analysed at 4 °C using amplified fragments of the *ompS1* regulatory region containing different lengths towards the 5′ terminus. They encompassed from +27 to either −310 (F1), −226 (F2), −164 (F3) or −114 (F4) (Fig. 5A). The corresponding F1-mt, F2-mt and F3-mt fragments contained the point mutations that diminished curvature (Fig. 1). In the presence of H-NS, the wild-type F1 fragment, which contains the whole regulatory region, shifted at 90 nM H-NS (Fig. 5B). In contrast, F1-mt shifted at 175 nM H-NS (Fig. 5B). Moreover, the shorter F2 and F3 fragments, which contain less of the 5′ upstream regulatory sequences, shifted at higher H-NS concentrations than F1: at 270 and 350 nM (Fig. 5B). The corresponding F2-mt and F3-mt fragments fully shifted at 350 and 435 nM H-NS (Fig. 5B). These data show that H-NS binding is favoured by the degree of curvature and illustrate the existence of further H-NS-binding sites upstream of the DNA curvature centre.

As a comparison, the F2 fragment was analysed by EMSA with StpA and LeuO (Fig. 5D, E). F2 shifted at 900 nM and F2-mt at 1100 nM StpA; both fragments shifted at 300 nM LeuO.

We have previously described an H-NS nucleation site in the 5′ regulatory region of *ompS1* within the LeuO (II) binding box (Fig. 6A). Mutations in this nucleation site cause derepression of *ompS1* expression in the absence of LeuO and a reduction in the H-NS binding affinity, and this site showed the highest affinity to H-NS by DNase footprinting analysis (De la Cruz *et al.*, 2007). Moreover, this site shows homology to the H-NS nucleation site for the *proU* gene (AATATATCGA) (Bouffartigues *et al.*, 2007). In contrast, mutations throughout the LeuO (I) binding box (Fig. 6A) did not have an effect on *ompS1* expression in the absence of LeuO, nor did they render an altered H-NS binding (De la Cruz *et al.*, 2007). These observations are in agreement with the experiment shown in Fig. 6B, where 50-mer double-stranded oligonucleotides encompassing the LeuO (II) or the LeuO (I) regions showed differential binding to H-NS. The LeuO (II) fragment was bound by H-NS and the LeuO (I) fragment did not bind (Fig. 6B). Most importantly, the LeuO (I) fragment contains the −151 and −135 residues of the curved region that were mutated to render either a lowering (mt) or a restoration (re) of the curvature (Fig. 1b). Thus, the curved region studied did not encompass the H-NS nucleation site.

These data are in accord with a model where the derepression observed with the pRO310-mt fusion (Fig. 2) is due to the lowering of the affinity of these two silencing proteins by the change in DNA curvature of the *ompS1* regulatory region, and with the notion that the effect of the introduced mutations is indeed on the DNA curvature and not on the alteration of the binding of H-NS to its nucleation site.

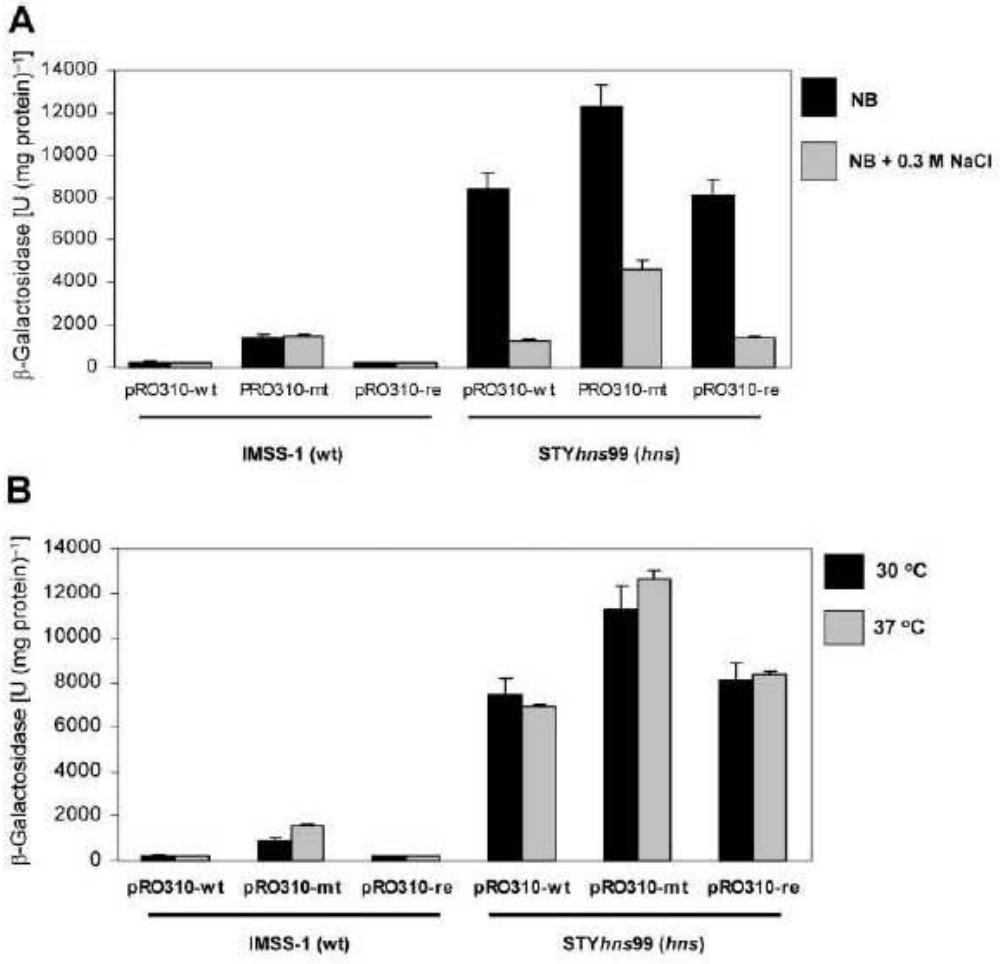

Fig. 3. Effect of *ompS1* intrinsic curvature upon growth under different osmolarity and temperature conditions. β-Galactosidase activity of the *lacZ* fusions pRO310-wt, -mt and -re was determined in *S.* Typhi wild-type and STY*hns*99 at low (NB) and high (NB + 0.3 M NaCl) osmolarity (A), and at 30 and 37 °C (B).

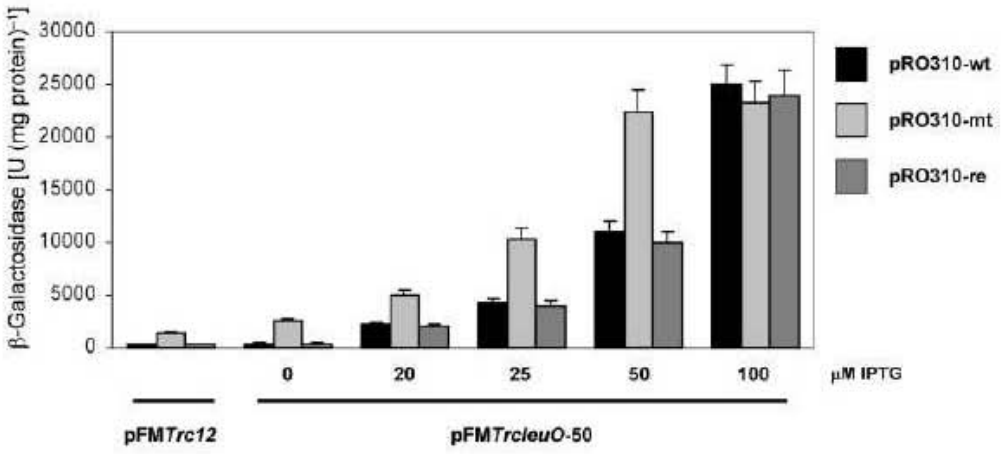

Fig. 4. Abolition of *ompS1* curvature favours derepression by LeuO: β-galactosidase activity of the *lacZ* fusions pRO310-wt, -mt and -re, in the presence of cloned LeuO on plasmid pFM*TrcleuO*-50 induced with increasing concentrations of IPTG. The pFM*Trc12* vector plasmid was included as control.

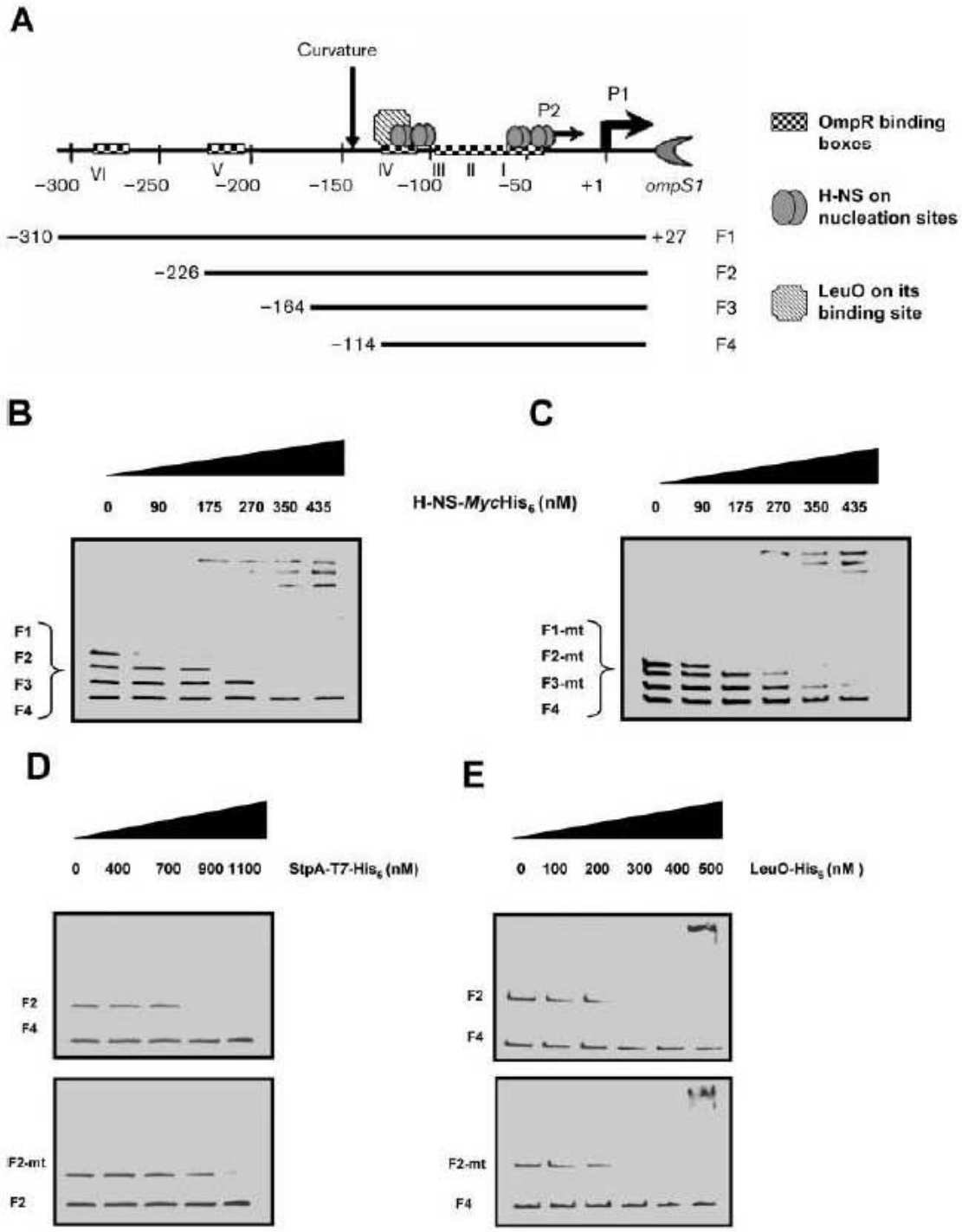

Fig. 5. Effect of DNA curvature on the binding of H-NS, StpA and LeuO to *ompS1*. (A) Schematic representation of the 5′ upstream regulatory region of *ompS1*, depicting the P1 and P2 promoters, and the binding sites for OmpR, H-NS and LeuO. The F1, F2, F3 and F4 fragments used in panels B, C, D and E are shown. (B–E) EMSAs for the binding of H-NS, StpA and LeuO to the F (wild-type) and the F-mt mutant fragment at different protein concentrations.

DISCUSSION

Transcription initiation is one of the most regulated steps of gene expression in bacteria. This process is mainly carried out by regulatory proteins that specifically recognize their DNA targets in a sequence-dependent fashion based on the interactions of the regulatory protein with the nucleotides. In addition, it has been demonstrated that primary DNA sequence is not the only recognized feature in the genome for the transcription regulatory process: the DNA topology has been shown to have a relevant role in

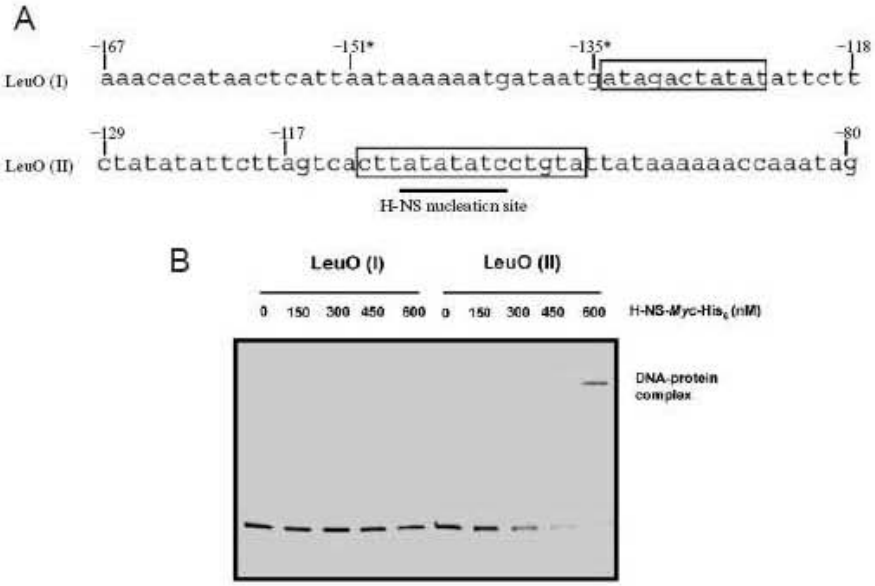

Fig. 6. Binding of H-NS to the nucleation site and to the curved region. (A) Sequence of the 50-mer double-stranded oligonucleotides encompassing the LeuO (I) and LeuO (II) binding boxes (outlined by rectangles). The coordinates for the upstream nucleotides are shown; the underline depicts the H-NS nucleation site (De la Cruz *et al.*, 2007). (B) EMSAs for the binding of H-NS to the 50-mer double-stranded oligonucleotides.

gene regulation (Ramani *et al.*, 1992; Falconi *et al.*, 1993; Pérez-Martín *et al.*, 1994; Jáuregui *et al.*, 2003; Poore & Mobley, 2003; Prosseda *et al.*, 2004; Olivares-Zavaleta *et al.*, 2006). Recently, the role of DNA curvature has been discussed for the promoters of the toxin and rRNA genes in *Bacillus anthracis* and *E. coli*, respectively (Hadjifrangiskou & Koehler, 2008; Pul *et al.*, 2008). For example, in the case of *virF*, encoding an activator of the AraC/XylS familiy, it was found that the intrinsic curvature acts as a thermo-switch, being flanked by two H-NS-binding sites forming a repressor loop (Prosseda *et al.*, 2004). A second example of a curvature-dependent transcription regulation has been reported for the *E. coli ompF* gene, where IHF has been found to bind upstream of its promoter, increasing the degree of DNA curvature, in a region relevant for negative regulation at high osmolarity (Ramani *et al.*, 1992).

A common approach taken to define the role of curved DNA regions in transcription regulation is based on serial deletions or replacements of the 5′ upstream regulatory region (Cobbett *et al.*, 1989; Falconi *et al.*, 1993; Asayama *et al.*, 2002; Kaji *et al.*, 2003; Prosseda *et al.*, 2004). This procedure introduces the considerable risk of modifying important regions that might not have been characterized yet. For this reason, in our study we employed a new and more precise approach that considers, in the first instance, an *in silico* analysis to identify the extent of DNA curvature at each position of the *ompS1* regulatory region. This was followed by site-directed mutagenesis on two specific nucleotides that reduce the extent of curvature (mt; Fig. 1B). Since both DNA intrinsic curvature and protein–DNA recognition are sequence-dependent events, changing the DNA sequence can alter both intrinsic DNA curvature and the sequence-dependent protein binding. For this reason, our study included two different and important internal controls. As our first internal control, we performed site-directed mutagenesis on the same two nucleotides as for mt in order to restore the curvature (re; Fig. 1B). It is worth mentioning that the computer design of our mutagenesis protocol provided the minimal number of changes in the DNA regulatory region. In addition, as our second internal control, we demonstrated by EMSAs, that the H-NS nucleation site is not present on the DNA regulatory region used in our point mutation analysis (LeuO (I) binding box; Fig. 6). Thus, the observations reported here are consistent with the concept that the effect of these nucleotide changes on the regulation of *ompS1* expression is due to the lowering of the DNA curvature in the region.

Using the wild-type and the aforementioned non-curved and curved mutagenized *ompS1* regulatory regions, we obtained *in vivo* and *in vitro* evidence that supports the role of a curved DNA sequence in the repression of *ompS1* expression. First, our theoretically predicted effects of the point mutation to diminish (mt) and restore (re) the intrinsic curvature of the *ompS1* regulatory region were verified by PAGE mobility analysis at 4 °C (Fig. 1). Secondly, our EMSA experiments demonstrated that the affinity of H-NS for the regulatory region is diminished following point mutagenesis that lowers the curvature and that there are H-NS binding sites upstream of the H-NS nucleation centre (Fig. 5). In further support of this notion, fragment F4 (−114 to +27), which contains only the H-NS nucleation site and no further upstream sequences, including the curved region, did not shift with H-NS. Similarly, StpA binding was reduced for the non-curved *ompS1* regulatory region (Fig. 5D) and, interestingly, LeuO affinity was not affected by the extent of curvature (Fig. 5E). Finally, there was a fivefold increase in the *ompS1* activity as assayed with a *lacZ* reporter fusion, upon removal of curvature in plasmid pRO310-mt (Fig. 2). This upregulation effect was not observed in our internal control of strains carrying plasmid pRO310-re with restored DNA curvature (Fig. 2).

All the results show that the curved region located at −151 to −135 in the *ompS1* regulatory region participates in the repression of *ompS1* transcription initiation. We previously accounted for the repression of *ompS1* expression by the formation of an H-NS nucleofilament (De la Cruz *et al.*, 2007). The new findings reported here now support a DNA-curvature-dependent bridging model that would account for full repression of *ompS1* expression (Fig. 7A), where the role of the curvature would be to facilitate the formation of DNA–H-NS–DNA bridges between downstream and upstream sites. Chromatin organization by loop domain formation conducive to DNA bridging has been discussed previously in detail (Dame *et al.*, 2005, 2006; Noom *et al.*, 2007; Dorman & Kane, 2009). In the F-mt fragments, where the curvature has been lowered by two point mutations, H-NS would still be binding, albeit at lower affinity, and could still cause some repression of expression by the formation of a nucleofilament-type structure (Figs 5B, C and 7B).

Moreover, the enhancement of LeuO activation upon removal of DNA curvature (Fig. 4) is consistent with our previous model of regulation for the *ompS1* porin gene in which LeuO acts as an antirepressor of H-NS and StpA (De la Cruz *et al.*, 2007), and where changes in DNA structural topology also affect repression. H-NS has been proposed to selectively silence a great number of horizontally acquired genes (Lucchini *et al.*, 2006; Navarre *et al.*, 2006) and also to act as a modulator of environmentally regulated gene expression (Atlung & Ingmer, 1997). H-NS modulates other important biological processes, such as DNA recombination, DNA replication and organization of the bacterial chromosome (Pérez-Martín *et al.*, 1994; Jáuregui *et al.*, 2003).

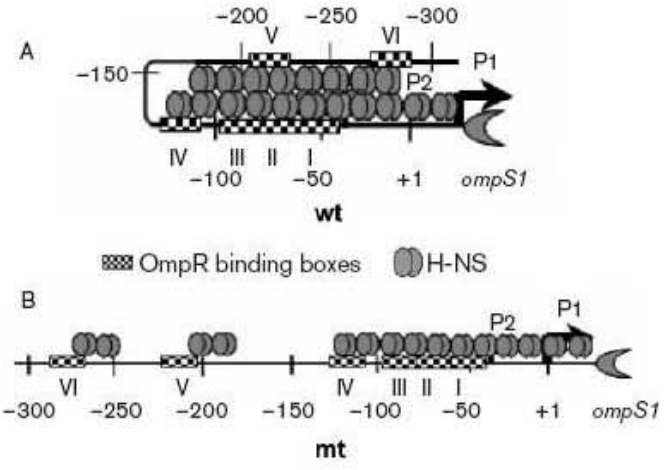

Fig. 7. DNA curvature and the binding of H-NS to the *ompS1* regulatory region. (A) Current model, which involves the formation of a DNA-curvature-mediated bridge structure that participates in the repression of *ompS1* expression in the wild-type. (B) Diminution of the DNA curvature in the mt mutant would favour the formation of an H-NS nucleofilament, where H-NS binds to DNA with lower affinity.

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6.3 Los sistemas de dos componentes EnvZ-OmpR y CpxA-CpxR regulan diferencialmente los promotores de *ompS1*

OmpR regula positivamente la expresión de *ompS1* actuando como un activador transcripcional que interactúa con la RNA polimerasa. OmpR modula la expresión de *ompS1* determinando el uso de los promotores: OmpR regula positivamente a P1 y negativamente a P2 (Oropeza *et al*., 1999; Flores-Valdez *et al*., 2003; De la Cruz *et al*., 2007). OmpR es el regulador de respuesta de un sistema de dos componentes que forma con EnvZ (Russo and Silhavy, 1991). EnvZ es una histidín cinasa que se autofosforila y transfiere su grupo fosfato a OmpR (OmpR-P) en el residuo aspártico 55 (Forst and Inouye, 1988; Mizuno and Mizushima, 1990; Pratt *et al*., 1996). En este trabajo evaluamos el efecto de la fosforilación de OmpR sobre la regulación de ambos promotores de *ompS1* y analizamos el papel de CpxR (un nuevo regulador) sobre la actividad del promotor P2.

El estado de fosforilación de OmpR, regula diferencialmente los promotores de *ompS1*. Decidimos evaluar el efecto de OmpR-P sobre la actividad de *ompS1*. Para esto, usamos dos plásmidos: pFM2000 y pFVD55A. El plásmido pFM2000 codifica para una proteína OmpR silvestre y pFVD55A codifica para la proteína OmpR con una mutación puntual en el aspártico 55 (D55A). Para evaluar la funcionalidad de nuestro plásmidos usamos la fusión traduccional *ompC-lacZ* que es estrictamente dependiente de OmpR-P. Evaluamos el efecto de *ompC* en el fondo silvestre (IMSS-1) y mutante *ompR* (IMSS-40) de *S*. Typhi. En el fondo silvestre se observó la expresión de *ompC*, no así, en la mutante *ompR* la actividad se apagó (Fig. 14A). Cuando esta mutante fue complementada con pFM2000, la expresión de *ompC* se restableció comparada a la silvestre. Contrariamente, cuando en esta mutante se introdujo el plásmido pFVD55A, la expresión de *ompC* no se restableció y tuvo el mismo perfil que la mutante *ompR* (Fig. 14A). Estos datos confirman la dependencia de *ompC* sobre OmpR-P y la funcionalidad de nuestros plásmidos.

Analizamos la expresión de *ompS1* usando la fusión pRO88 que carece de los elementos de regulación negativa y contiene las regiones de regulación positiva mediados por OmpR. En el fondo silvestre la expresión de pRO88 fue alta, mientras que en la ausencia de OmpR la expresión de la fusión fue reducida a la mitad (Fig. 14A). Cuando la mutante *ompR* se complementó con pFM2000 la expresión fue restablecida comparada a la silvestre y con la introducción del plásmido pFVD55A la actividad no restituyó los máximos niveles (Fig. 14A).

Para analizar detalladamente el efecto de OmpR-P sobre la actividad de los dos promotores de *ompS1* realizamos experimentos de Primer Extensión usando RNA de *ompS1* proveniente del plásmido pRO88. En el fondo silvestre, la expresión de *ompS1* se originó a partir de P1 (Fig. 14B). En ausencia de OmpR, la expresión fue dependiente de P2 (Fig. 14B). Cuando esta mutante se complementó con pFM2000, la expresión de *ompS1* volvió a ser dependiente de P1; contrariamente con pFVD55A la expresión fue dependiente de P2 (Fig. 14B). Estos datos indicaron que el estado de fosforilación de OmpR reguló diferencialmente ambos promotores de *ompS1*. OmpR-P reguló positivamente a P1 y negativamente a P2, en otras palabras la actividad de P1 es dependiente de OmpR fosforilado, mientras que P2 es activo solamente cuando OmpR no está fosforilado.

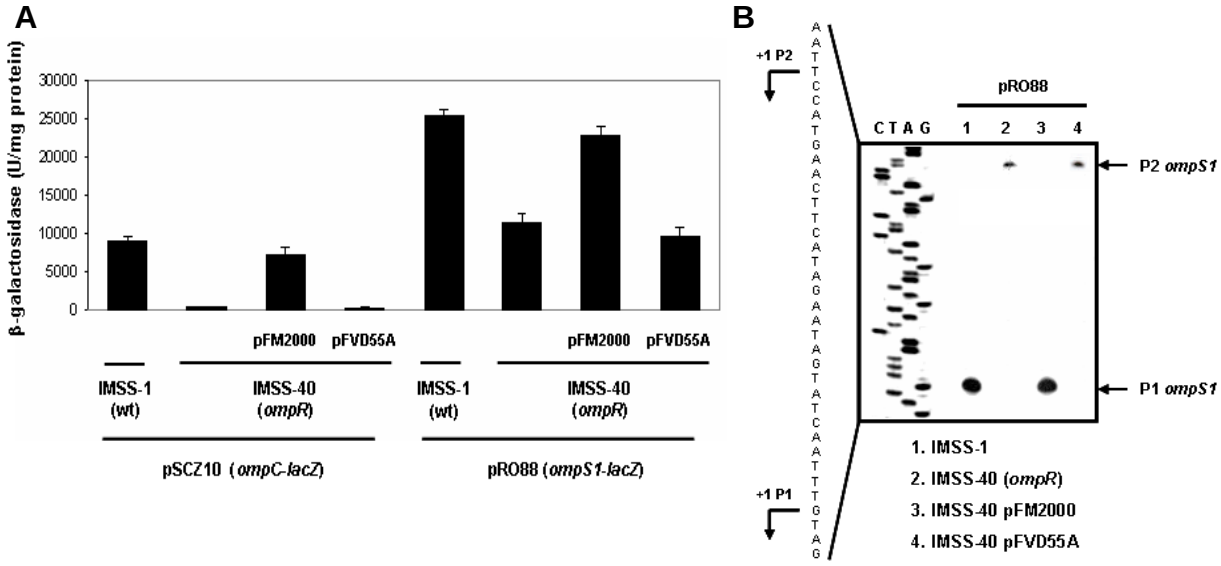
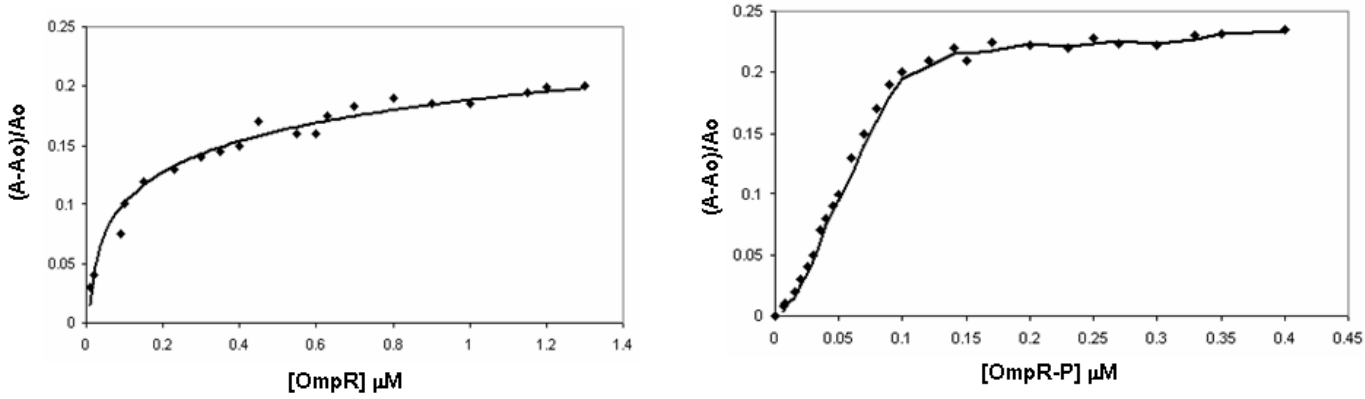


Fig. 14. OmpR-P regula diferencialmente ambos promotores de *ompS1*. A) Ensayos de β-galactosidasa fueron evaluados con la fusión *ompC-lacZ* y *ompS1-lacZ*. En el fondo silvestre de *S*. Typhi (IMSS-1) y mutante *ompR* (IMSS-40) complementadas con OmpR wt (pFM2000) y OmpR mutante en el sitio de fosforilación (pFVD55A). B) Ensayo de primer extensión para detectar el inicio de la transcripción usando RNA de *ompS1* proveniente de la fusión pRO88 en el fondo silvestre de *S*. Typhi (IMSS-1) y mutante *ompR* (IMSS-40) complementadas con OmpR wt (pFM2000) y OmpR mutante en el sitio de fosforilación (pFVD55A)

La fosforilación de OmpR aumenta dramáticamente la afinidad de unión a la región promotora de *ompS1*. Debido a que el estado de fosforilación de OmpR determinó la regulación diferencial de los promotores de *ompS1*, decidimos evaluar el efecto de la fosforilación de OmpR sobre la unión al DNA. Para esto se hicieron ensayos de anisotropía de fluorescencia donde se marcaron oligonucléotidos complementarios de doble cadena con fluoresceína y se observó el cambio en la fluorescencia al unirse la proteína al DNA. Estos oligonucleotidos de 71 pb abarcan la región promotora de *ompS1*. La proteína OmpR se incubó con acetil-fosfato para generar OmpR-P. OmpR y OmpR-P se incubaron con el DNA y se midieron las afinidades relativas (Fig. 15). La constante de disociación para la unión de OmpR

sobre *ompS1* fue de 157.3 nM. Cuando OmpR se fosforiló, la constante de disociación fue de 2.2 nM (Fig. 15). Lo anterior nos indicó que OmpR-P era 72 veces más afín a la región promotora de *ompS1* que OmpR; esta afinidad era mayor a lo reportado para los genes *ompC* y *ompF* (Head *et al*., 1999).



DNA	OmpR Kd (nM)	OmpR-P Kd (nM)	$\frac{Kd_{OmpR}}{Kd_{OmpR-P}}$
I-II-III (*ompS1*, 71 pb)	157.3	2.2	72
C1-C2-C3 (*ompC* , 70 pb)*	496.5	15.4	33
F1-F2-F3 (*ompF* , 67 pb)*	86.7	31.4	3

*Datos tomados de Head *et al.*, 1998

Fig. 15. OmpR-P tiene mayor afinidad a la región promotora de *ompS1* que OmpR. Experimentos de anisotropía de fluorescencia determinando las constantes de disociación de OmpR y OmpR-P sobre la región promotora de *ompS1*. Se muestran las constantes de disociación de OmpR y OmpR-P sobre *ompS1* y se compara con lo reportado para *ompC* y *ompF*

EnvZ es la principal cinasa que fosforila a OmpR para la regulación diferencial de ambos promotores de *ompS1*. EnvZ fosforila a OmpR. Sin embargo, se ha reportado que pequeñas moléculas como el acetil-fosfato pueden servir como donadores de fosfato y fosforilar a OmpR independientemente de EnvZ (Hsing and Silhavy 1997; Matsubara and Mizuno 1999; Bang *et al*., 2000). Para determinar si la forma fosforilada de OmpR era dependiente de la actividad cinasa de EnvZ, evaluamos la expresión de *ompS1* (pRO88) en un fondo mutante *ompB* (*ompR-envZ*) (IMSS-81), en donde EnvZ y OmpR están ausentes. La expresión de *ompS1* en la mutante *ompB* disminuyó a la mitad y fue similar al efecto visto con la mutante *ompR* (Fig. 16A). Cuando el plásmido pFM2000 complementó a la mutante *ompR*, la expresión de *ompS1* no se restableció como la silvestre (Fig. 16A).

Se realizaron ensayos de PE para analizar la actividad de los promotores de *ompS1* en ausencia de OmpR y EnvZ (Fig. 16B). En un fondo mutante *ompB*, la

expresión de *ompS1* fue dependiente de P2. Aun cuando esta mutante llevó el plásmido pFM2000, no fue capaz de complementar la actividad con respecto a la silvestre (Fig. 16B).Esto nos indicaba que EnvZ se requiere como la principal cinasa que fosforila a OmpR, ya que en ausencia de ésta, la expresión del promotor P1 que es dependiente de OmpR-P se abatió, mientras que el promotor P2 se activó, ya que se expresó en ausencia de OmpR-P.

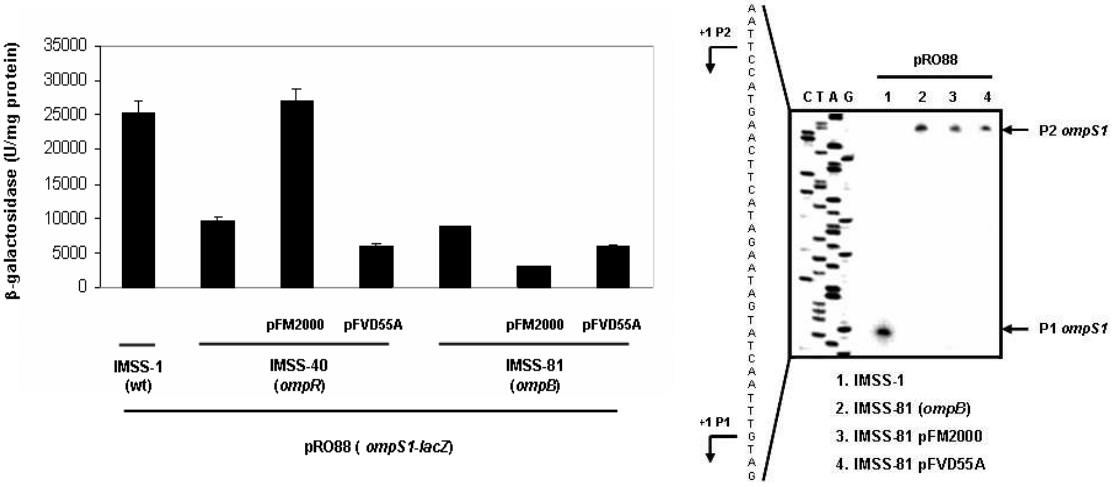


Fig. 16. En ausencia de EnvZ, la expresión de *ompS1* depende del promotor P2 independientemente de OmpR. A) Experimentos de β-galactosidada con la fusión *ompS1-lacZ* en mutantes *ompR* (IMSS-40) y *ompB* (*ompR-envZ*) (IMSS-81) complementadas con OmpR wt (pFM2000) y OmpR mutante en el sitio de fosforilación (pFVD55A). B) Ensayo de primer extensión para detectar el inicio de la transcripción usando RNA de *ompS1* proveniente de la fusión pRO88 fondo mutante *ompR* (IMSS-40) y mutante *ompB* (IMSS-81) complementadas con OmpR wt (pFM2000) y OmpR mutante en el sitio de fosforilación (pFVD55A).

CpxR regula positivamente la expresión del promotor P2 en ausencia de OmpR-P. CpxR es un regulador de respuesta que junto a CpxA forma un sistema de dos componentes de transducción de señales. Se ha descrito que el sistema CpxRA co-regula muchos genes con OmpR, por ejemplo el caso de los genes de porinas *ompC* y *ompF* (Batchelor *et al*., 2005). Para analizar el probable papel de CpxR, analizamos la expresión de *ompS1* en un fondo Δ*cpxR* y Δ*ompR* Δ*cpxR* usando la fusión pRO88. En una mutante Δ*cpxR* la expresión de *ompS1* fue similar a la cepa silvestre, sin embargo, en la doble mutante Δ*ompR* Δ*cpxR* la expresión de *ompS1* disminuyó significativamente comparada a la mutante sencilla Δ*ompR* (Fig. 17A). Para corroborar dicho efecto, se usó la fusión pRO310 que contiene todos los elementos de regulación, tanto positivos como negativos para el control de *ompS1*. Debido a que esta fusión tiene niveles de expresión muy bajos, se indujo el regulador LeuO, el cual antagoniza el efecto represor de H-NS y StpA sobre *ompS1* (De la Cruz *et al*., 2007). Como se observó para la fusión pRO88, en ausencia de CpxR los niveles de expresión de pRO310 fueron similares a la cepa silvestre (~25,000 U), indicando que la ausencia

de CpxR no afecta la expresión de *ompS1*. En la doble mutante Δ*ompR* Δ*cpxR* los niveles de la fusión pRO310 fueron más bajos comparados a la mutante Δ*ompR* (Fig. 17B). Haciendo preparaciones de proteínas de membrana externa induciendo el regulador LeuO, la porina OmpS1 fue casi indetectable en su expresión cromosomal en un fondo Δ*ompR* Δ*cpxR* (Fig. 17C), de acuerdo a los datos de las fusiones reporteras. Con base a lo anterior, se analizó el efecto de CpxR sobre los promotores de *ompS1*. La ausencia de CpxR no alteró el uso de los promotores, donde la expresión de *ompS1* fue dependiente del promotor P1, debido a la presencia de OmpR-P. En una mutante Δ*ompR*, la expresión de *ompS1* proviene del promotor P2, sin embargo, en una doble mutante Δ*ompR* Δ*cpxR* la actividad del promotor P2 desapareció (Fig. 17D). Estos datos indican fuertemente que CpxR positivamente reguló la expresión de *ompS1*, activando el promotor P2 en ausencia de OmpR-P.

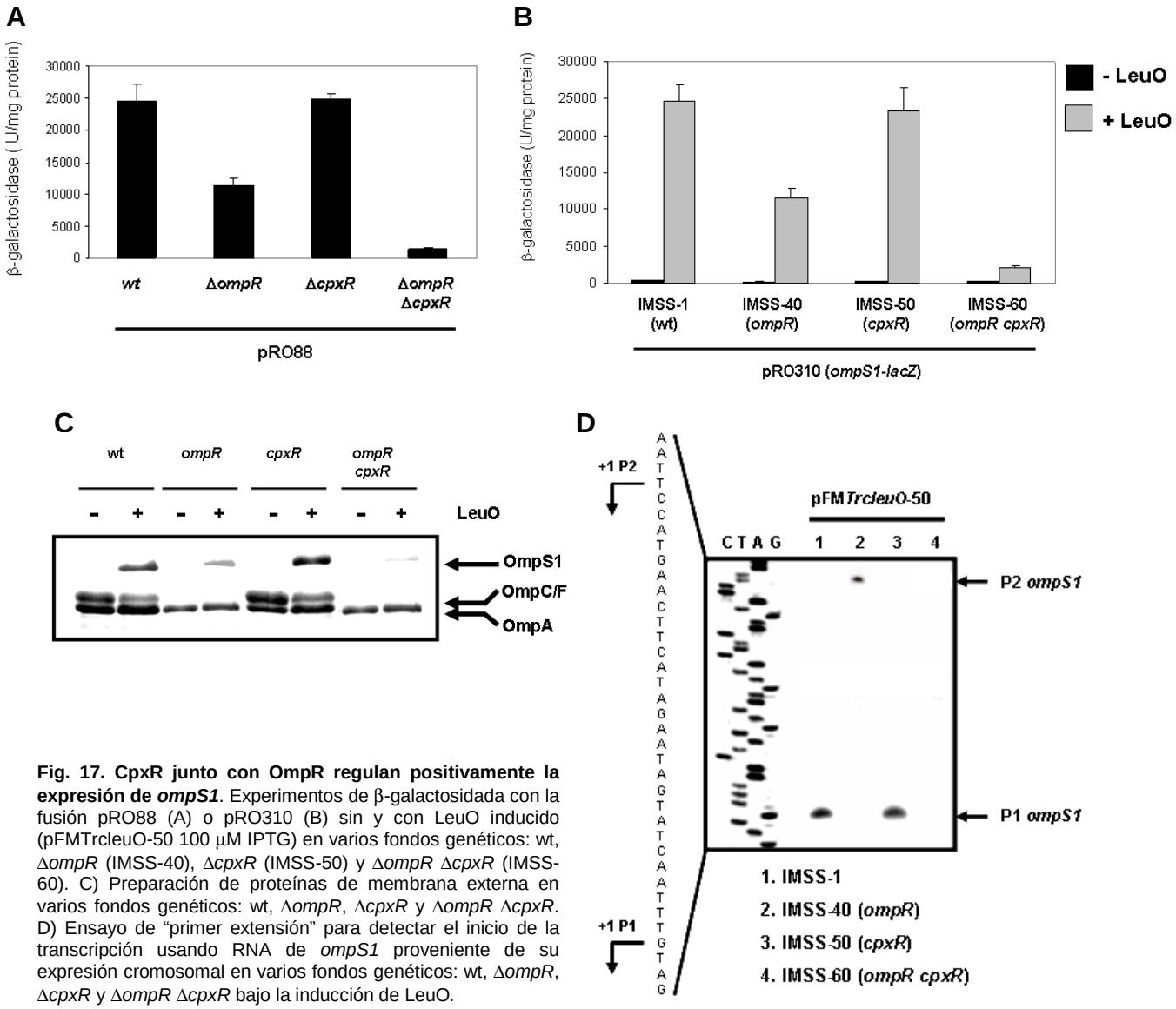


Fig. 17. CpxR junto con OmpR regulan positivamente la expresión de *ompS1*. Experimentos de β-galactosidada con la fusión pRO88 (A) o pRO310 (B) sin y con LeuO inducido (pFMTrcleuO-50 100 μM IPTG) en varios fondos genéticos: wt, Δ*ompR* (IMSS-40), Δ*cpxR* (IMSS-50) y Δ*ompR* Δ*cpxR* (IMSS-60). C) Preparación de proteínas de membrana externa en varios fondos genéticos: wt, Δ*ompR*, Δ*cpxR* y Δ*ompR* Δ*cpxR*. D) Ensayo de "primer extensión" para detectar el inicio de la transcripción usando RNA de *ompS1* proveniente de su expresión cromosomal en varios fondos genéticos: wt, Δ*ompR*, Δ*cpxR* y Δ*ompR* Δ*cpxR* bajo la inducción de LeuO.

CpxR regula directamente la expresión de *ompS1*. CpxR activa el promotor P2 de *ompS1*. Usando la secuencia consenso de DNA reportada a la que se une CpxR en *E. coli* (De Wulf *et al*., 2002), encontramos un sitio de unión probable entre las cajas II y III de unión a OmpR. Para determinar si CpxR se unía directamente a la región reguladora de *ompS1*, se realizaron ensayos tipo EMSA. La proteína CpxR se purificó e incubó con el fragmento F1 (-310 a +27). En presencia de CpxR, el fragmento F1 se retardó completamente a 200 nM (Fig. 18A). Para determinar cuantitativamente la afinidad de unión de CpxR hacia la región promotora de *ompS1* se diseñaron experimentos tipo ELISA. Usamos oligonucléotidos (66 nt) de doble cadena que comprendió la región promotora de *ompS1* incluyendo el sitio de unión probable para CpxR. Agregando cantidades crecientes de CpxR, fue determinada una *Kd* aparente de 67.5 nM (Fig. 18B). Estos datos indicaron que CpxR directamente se unió a la región reguladora de *ompS1* activando el promotor P2.

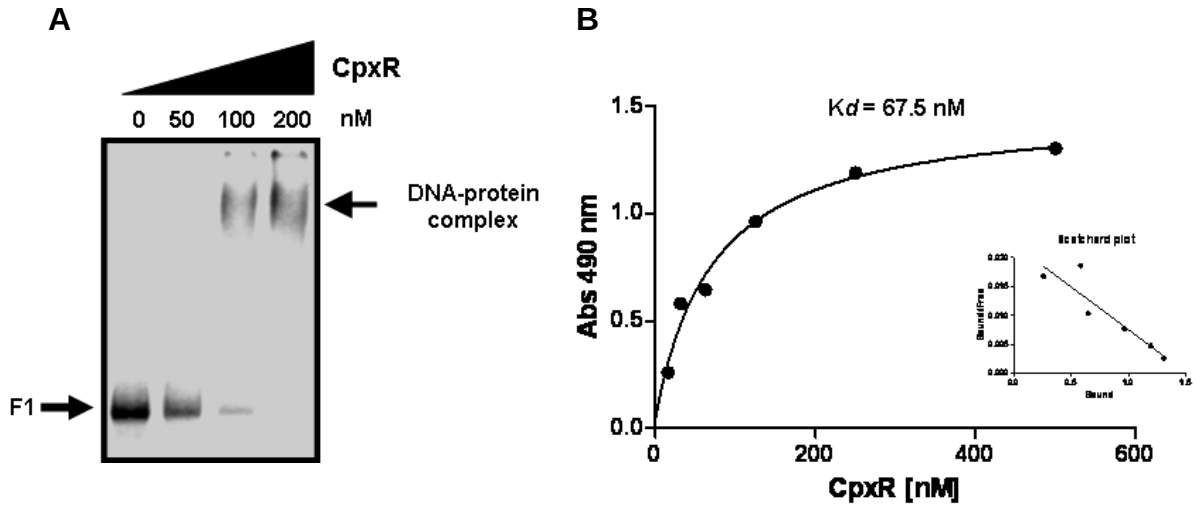


Fig. 18. CpxR se une directamente a la región reguladora de *ompS1*. A) Experimentos tipo EMSA usando el fragmento F1 que comprende la región reguladora de *ompS1* (-310 a +27) con diferentes cantidades de la proteína His_6-CpxR-GFP. B) Experimentos tipo ELISA determinando cuantitativamente de la afinidad de unión de CpxR sobre la región promotora de *ompS1*.

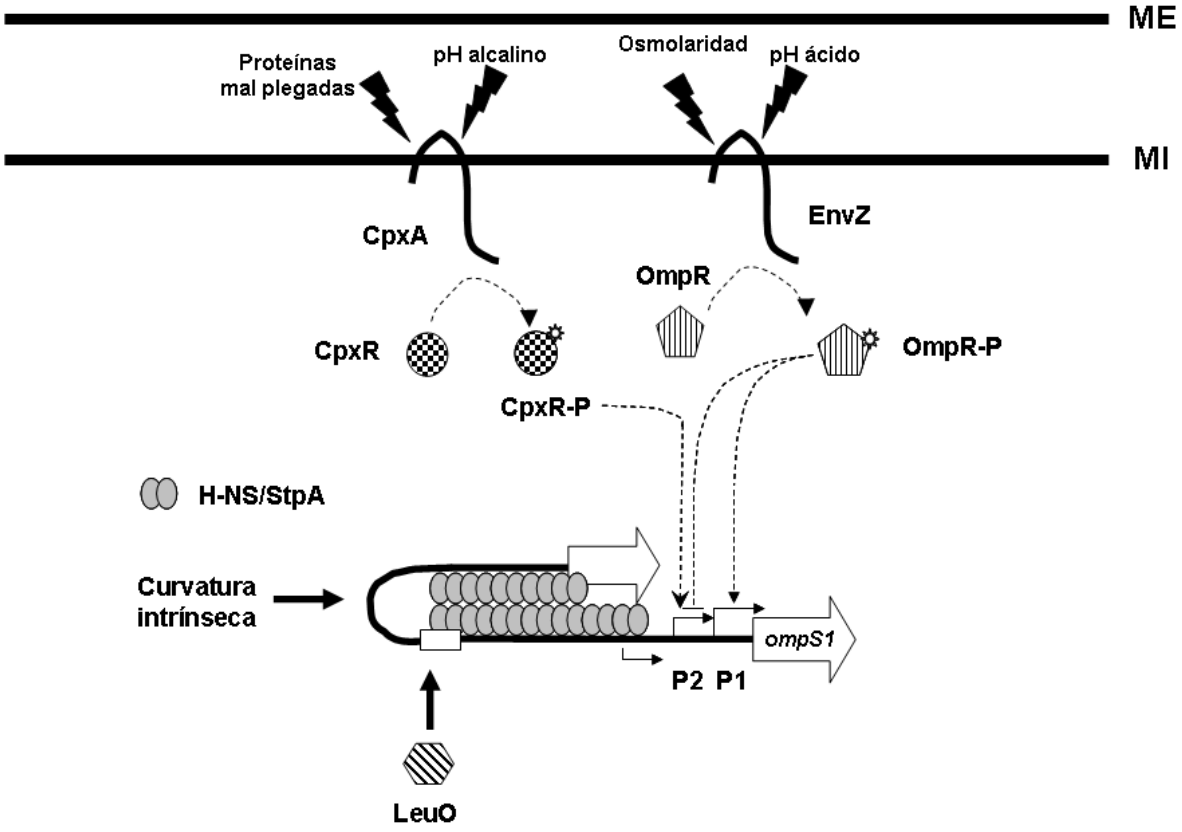


Fig. 19. Modelo regulatorio del gen *ompS1* en *Salmonella*. H-NS/StpA reprimen la expresión de *ompS1* formando una estructura tipo horquilla, donde la curvatura intrínseca del DNA favorece dicha estructura represora. Además, H-NS/StpA pueden extenderse formando un nucleofilamento silenciando los promotores de *ompS1* (P1 y P2). LeuO actúa como un antagonista de H-NS y StpA, compitiendo por un sitio en el DNA (rectángulo blanco), desestabilizando el complejo nucleorepresor y desplazándolos de la región reguladora de *ompS1.* Cuando H-NS/StpA son desplazados por LeuO, OmpR-P se une a la región promotora de *ompS1* activando el promotor P1 y por sobreposición reprime al promotor P2. En ausencia de OmpR-P, el promotor P2 es activado por CpxR-P.

7. DISCUSIÓN

La porina OmpS1 es homóloga a las porinas mayoritarias OmpC/OmpF. Sin embargo, a diferencia de éstas, la expresión de *ompS1* es quiescente. Trabajos anteriores realizados en el laboratorio indicaron dos conceptos muy importantes: los bajos niveles de expresión están mediados por secuencias en *cis* donde H-NS reprime directamente y, la expresión de *ompS1* se activó por dos promotores controlados por OmpR (Oropeza *et al*., 1999; Flores-Valdez *et al*., 2003). A pesar de que H-NS silenciaba la expresión de *ompS1*, una mutante STY*hns*99 no desreprimió completamente la expresión de la fusión pRO310 (fusión mas larga) comparada la fusión pRO88 (fusión que carece de los elementos de regulación negativa) sugiriendo la presencia de otro represor que actuaba en ausencia de H-NS. La proteína H-NS pertenece a un grupo denominadas nucleoides por: su abundancia en la célula, sus efectos pleiotrópicos y su papel en estructurar el cromosoma bacteriano. Dentro de este grupo se encuentra la proteína StpA cuya expresión es relativamente baja comparada a la proteína H-NS. Los principales fenotipos asociados a StpA se habían descritos en ausencia de H-NS. En *E. coli* uropatógena, el perfil transcriptómico en una doble mutante *hns stpA* fue diferente a la mutante sencilla *hns*, donde la mutante sencilla *stpA* no mostró algún fenotipo sobre estos genes (Müller *et al*., 2006). En *E. coli* enterotoxigénica, la ausencia de H-NS y StpA desreprimió más la expresión del sistema de secreción tipo II comparada a la ausencia de H-NS solamente (Yang *et al*., 2007).

En términos de la regulación de *ompS1*, nuestra hipótesis indicaba que otro regulador negativo reprimía su expresión. Concretamente encontramos que StpA reguló negativamente la expresión de *ompS1* en una mutante *hns*, indicando que en este caso al igual que los anteriores, StpA actúa como un soporte molecular de H-NS (De la Cruz *et al*., 2007). Un trabajo reciente mostró que StpA si tiene un efecto en presencia de H-NS controlando la expresión de un grupo de genes en *S*. Typhimurium, que son reprimidos por H-NS y predominantemente están bajo el control positivo de RpoS, CRP y PhoP, principalmente en la fase logarítmica de crecimiento. La expresión de *stpA* respondió a cambios en los niveles de glucosa en la célula por lo que estos últimos hallazgos hacen ver a StpA como un regulador involucrado en el flujo de nutrientes (Lucchini *et al*., 2009).

En términos de reguladores positivos que activaran la expresión de *ompS1*, OmpR era la única proteína descrita (Oropeza *et al*., 1999). Sin embargo, a pesar que OmpR está presente en la célula, los niveles de expresión de *ompS1* son muy bajos y no se puede detectar en una preparación de proteínas de membrana externa a

comparación de las porinas OmpC/OmpF. Debido a que H-NS/StpA fuertemente silencian la expresión de *ompS1*, un probable antagonista debía de funcionar en la célula para contrarrestar la represión ejercida por esas proteínas nucleoides. LeuO es un regulador de la familia LysR que había sido reportado como un antagonista de H-NS en la autorregulación de *leuO* (Chen *et al*., 2001; 2003; Chen and Wu, 2005) y en la desrepresión del operon *bgl* (Ueguchi *et al*., 1998). En nuestro grupo se encontró que LeuO junto con OmpR activaba la expresión de *ompS2*, un gen que codifica para otra porina quiescente (Fernández-Mora *et al*., 2004). Cuando LeuO se indujo, desreprimió completamente los niveles de expresión de *ompS1*. Este efecto desrepresor es dependiente de la concentración: bajos niveles de LeuO activaron la expresión de *ompS2* mientra que *ompS1* fue reprimido, y altos niveles de LeuO reprimieron *ompS2* y activaron la expresión de *ompS1* (De la Cruz *et al*., 2007). Esta expresión diferencial sería similar a la regulación recíproca de OmpC y OmpF mediada por lo niveles de OmpR-P. Los datos generados en este aspecto sirvieron como base para el análisis proteómico del regulón LeuO en *S*. Typhi, donde además de OmpS1 y OmpS2, se encontraron 2 nuevas proteínas inducidas (AssT, STY3070) y 3 reprimidas (OmpX, Tpx y STY1978) (Hernández-Lucas *et al*., 2008). Donde la función de estos genes no está relacionada, fue propuesto a LeuO como un regulador global en *S*. Typhi. Recientemente se reportó en *E. coli*, que LeuO controla la expresión de genes involucrados en resistencia a drogas sulfa (Shimada *et al*., 2009). Estos datos empiezan a elucidar el importante papel de LeuO en la fisiología y patogénesis bacteriana.

Estas observaciones estaban a favor de la hipótesis planteada donde la célula tiene un programa de desrepresión sobre el gen *ompS1*. En términos de la regulación positiva de LeuO sobre *ompS1*, encontramos que este regulador tipo LysR actuó como antagonista de las proteínas nucleoides H-NS y StpA. LeuO desreprimió los dos promotores de *ompS1* (De la Cruz *et al*., 2007), corroborando su efecto antagonista porque ya había sido descrito que ambos promotores eran silenciados por H-NS (Flores-Valdez *et al*., 2007). Haciendo un análisis detallado del papel silenciador de H-NS sobre la expresión de *ompS1*, encontramos dos regiones que eran necesarias para su unión. Una de esas regiones se encontraba alrededor de la caja IV de unión a OmpR, donde la remoción de esta zona en fusión pRO310 incrementaba significativamente la expresión de *ompS1* (Oropeza *et al*., 1999). En esta región fue localizado un sitio de nucleación de H-NS donde se encontró una secuencia (AATATATCGA) que ha sido reportada como consenso para H-NS (Fig. 7; Bouffartigues *et al*., 2007; Lang *et al*., 2007). Interesantemente, LeuO se unió entre -137 a -96 en dos cajas: LeuO I (-134 a -125) y LeuO II (-113 a -99) (De la Cruz *et al*.,

2007). El sitio LeuO II se sobrepone al sitio de nucleación ubicado de -104 a -110. Mediante ensayos de EMSA competitivo, LeuO desplazó a H-NS y StpA de la región reguladora de *ompS1* (De la Cruz *et al*., 2007). Estos datos apuntan a un mecanismo donde LeuO contrarrestó la represión ejercida por H-NS compitiendo por un sitio en la región reguladora de *ompS1* donde ambos se sobrepusieron (LeuO II/sitio de nucleación de H-NS). LeuO actuó solamente como un desrepresor permitiendo que OmpR, un activador transcripcional clásico, activara la expresión de *ompS1* (De la Cruz *et al*., 2007). Este mecanismo de regulación contrastó con el modelo donde LeuO bloqueaba físicamente el progreso de H-NS en la autorregulación de *leuO* (Fig. 9; Chen *et al*., 2001; 2003; Chen and Wu, 2005). Este nuevo modelo abría una nueva perspectiva de los diferentes mecanismos de acción que puede usar LeuO para contrarrestar la represión ejercida por H-NS. Este nuevo mecanismo sería similar a lo reportado para otros antagonistas de H-NS, como es el caso de Ler, RovA y VirB (Bustamante *et al*., 2001; Heroven *et al*., 2004; Tuner and Dorman, 2007). A pesar que se ha descrito que el ppGpp y el ayuno estimulan la expresión de *leuO* usando el promotor p*leu-500* en un sistema reportero plasmídico (Fang and Wu, 1998; Fang *et al*., 2000; Majumder *et al*., 2001), aún no es claro que señales ambientales estimulan su expresión en un contexto cromosomal, y si existe alguna molécula que esté presente en el hospedero que induzca su actividad transcripcional, ya que se ha descrito que el gen *leuO* participa en la patogénesis de *Salmonella* (Tenor *et al*., 2004; Lawley *et al*., 2006; Rodríguez-Morales *et al*., 2006; Chaudhuri *et al*., 2009).

Además del papel de reguladores transcripcionales, se ha reportado que la curvatura intrínseca del DNA controla la expresión de ciertos genes favoreciendo la unión de activadores o represores transcripcionales a las regiones reguladoras. En el caso especifico de H-NS, regiones con curvaturas intrínsecas en el DNA favorecen la formación de estructuras tipo puente, donde se forman horquillas represoras silenciando los promotores (Fig. 8; Noom *et al*., 2007; Dorman and Kane, 2009). Un ejemplo clásico del papel de la curvatura sobre la regulación de genes de virulencia, es el caso del gen *virF*, que codifica para un regulador transcripcional involucrado en la patogénesis de *Shigella flexneri* donde la curvatura funcionó como un termosensor, permitiendo la represión de H-NS a bajas temperaturas (Prosseda *et al*., 2004). Para *ompS1*, encontramos que la represión ejercida por H-NS sobre el gen es favorecida por la presencia de curvatura intrínseca localizada de -151 a-135 con respecto a P1, donde existen zonas de unión a H-NS tanto corriente arriba como corriente abajo permitiendo la formación de una estructura tipo puente u horquilla represora (Fig. 8 y 19; De la Cruz *et al*., 2009). La disminución de la curvatura intrínseca en esta zona (mt) tuvo un efecto desrepresor en la expresión de *ompS1*, no permitiendo que H-NS

se uniera con la misma afinidad comparada a la región que presentaba curvatura intrínseca (wt) (De la Cruz *et al*., 2009). Estos datos apuntan a un modelo complejo, donde son necesarias tanto interacciones transcripcionales proteína-DNA como elementos intrínsecos dados por la secuencia nucleotídica de la región reguladora de *ompS1* (Fig. 19).

El control de la expresión de *ompS1* está bajo el control de dos promotores: P1 y P2. Analizando el papel de la fosforilación de OmpR se encontró que OmpR-P activaba la expresión del promotor P1 y reprimía la actividad del promotor P2 (Fig. 15). La fosforilación de OmpR incrementó dramáticamente su afinidad de unión a DNA a la región promotora de *ompS1* (72 veces) (Fig. 16). La fosforilación de OmpR fue dependiente de la presencia de EnvZ, la cinasa que fosforila a OmpR para la regulación diferencial de ambos promotores de *ompS1* (Fig. 17). En términos del control transcripcional del promotor P2, sólo había sido reportado que OmpR-P lo regulaba negativamente. La hipótesis planteada en este trabajo establecía que el promotor P2 de *ompS1* estaría bajo el control positivo de otro regulador diferente a OmpR. CpxR que junto a CpxA forma un sistema de dos componentes involucrado en la respuesta bacteriana hacia el estrés en la envoltura celular, reguló positivamente la expresión del promotor P2 de *ompS1*, estando en línea a la hipótesis planteada. La regulación mediada por CpxR sobre el promotor P2 fue en ausencia de OmpR, donde CpxR actuó como un soporte transcripcional para mantener la expresión *ompS1* vía P2 (Fig. 18). CpxR se unió directamente a la región reguladora de *ompS1* encontrándose una constante de disociación aparente de 65 nM (Fig. 19). En términos de sitio consenso para CpxR en *E. coli* (GTAAA-N_5-GTAAA) (De Wulf *et al*., 2005), se encontró una secuencia GAAAC-AAATT-GAAAT ubicada entre las cajas III y II de unión a OmpR. Esta sobreposición de OmpR y CpxR sobre el DNA también fue encontrada para los genes de porinas *ompC*/*ompF* (Batchelor *et al*., 2005). El sitio de CpxR sobre *ompS1* es homólogo al sitio putativo encontrado para el operón *csgBAC* (GTAAA-AAATT-GTCCA) que es regulado por CpxR (Prigent-Combaret *et al*., 2001; De Wulf *et al*., 2005). Además de regular los genes de porinas, OmpR y CpxR parecen co-regular varios genes como *nanC*, *csgD*, *inv* y *ssrAB* (Condemine *et al*., 2005; Jubelin *et al*., 2005; Carlsson *et al*., 2007; Brzosteck *et al*., 2007; De la Cruz *et al*., datos no publicados). Estos datos podrían indicar que estos reguladores evolutivamente se han seleccionado para funcionar sobre blancos comunes, como es el caso de *ompS1*.

Probablemente el uso de ambos promotores de *ompS1* responda a diferentes nichos ecológicos de *Salmonella*. Los sistemas de dos componentes EnvZ-OmpR y CpxA-CpxR son reguladores involucrados en la virulencia de *Salmonella enterica*

(Dorman *et al*., 1989; Humphreys *et al*., 2004). En patogénesis, OmpR-P además de regular el repertorio de porinas, activa la expresión de SPI-2 vía *ssrAB*, confiriendo a *Salmonella enterica* la capacidad de sobrevivir y replicarse dentro de los macrófagos (Lee *et al*., 2000; Hansen-Wester and Hensel 2003; Waterman and Holden 2003). El promotor P1 de *ompS1* sería activo en esas condiciones donde OmpR-P activa este promotor. La activación de P1 dentro del macrófago está de acuerdo con datos que muestran la expresión de *ompS1* dentro del ratón (Lawley *et al*., 2006). El sistema Cpx ha sido involucrado en la regulación de SPI-1 (Nakayama *et al*., 2003), requerida para invadir células epiteliales. En este aspecto, una mutante *ompS1* muestra una virulencia atenuada, principalmente por vía oral en el modelo del ratón (Rodríguez-Morales *et al*., 2006), donde probablemente el promotor P2 actuaría en condiciones extracelulares como es el caso de la invasión, donde OmpR-P no es requerido. Otra hipótesis involucraría a P2 en algunas condiciones ambientales como vida libre (suelo o agua) donde OmpR no estuviese fosforilado permitiendo la actividad de este promotor.

Estos conceptos indicarían que el promotor P2 actúa como un soporte transcripcional para P1, donde en la ausencia de OmpR-P, *S*. Typhi hace uso de un segundo promotor a diferencia de *ompC* y *ompF* que son estrictamente dependientes de OmpR-P. Este sistema muestra los mecanismos transcripcionales usados por *Salmonella* para mantener el repertorio de porinas (De la Cruz and Calva 2010), como es el caso de la expresión de OmpS1 mediada por CpxR aún en ausencia del regulador maestro OmpR-P.

En conclusión, el trabajo de esta tesis ilustra la complejidad de la regulación genética de las porinas, en particular de una porina quiescente como lo es OmpS1 (Fig. 19), y sirve de base para el estudio de nuevas preguntas sobre los mecanismos de activación y represión transcripcional de las porinas. Algunas de estas preguntas y temas de estudio a futuro se han ilustrado en nuestra reciente revisión (De la Cruz and Calva 2010).

8. CONCLUSIONES

Durante mi formación doctoral trabajando con la regulación del gen de porina *ompS1* en *S*. Typhi, se obtuvieron las siguientes conclusiones:

- StpA reprimió directamente la expresión de *ompS1* en un fondo mutante *hns*.
- LeuO actuó como un antagonista de H-NS/StpA desreprimiendo la expresión de *ompS1*.
- LeuO se unió alrededor de la caja IV de unión a OmpR, sobreponiéndose a un sitio de nucleación para H-NS.
- LeuO desplazó a H-NS/StpA de la región reguladora de *ompS1* y no bloqueó físicamente el progreso de H-NS.
- La curvatura intrínseca localizada entre -151 a -135 participó en la represión transcripcional de *ompS1*.
- La curvatura intrínseca permite la formación de una horquilla represora conformada por complejos H-NS-DNA-H-NS, entre el sitio de nucleación localizado alrededor de la caja IV de unión a OmpR y secuencias arriba del centro de la curvatura.
- La fosforilación de OmpR determina el uso de los promotores de *ompS1*: OmpR-P activó y reprimió a P1 y P2, respectivamente.
- La fosforilación de OmpR incrementó dramáticamente (72 veces) su afinidad a la región promotora de *ompS1*.
- CpxR activó el promotor P2 en ausencia de OmpR-P, uniéndose directamente a la región promotora de *ompS1*.

La mayor parte de los datos arriba mencionados se publicaron en dos artículos (De la Cruz *et al*., 2007, 2009); y el remanente se encuentran en un manuscrito en preparación y se detallan en el texto de esta tesis. Estas conclusiones son esquematizadas en el modelo mostrado en la figura 19. Adicionalmente, se publicó un artículo de revisión sobre la regulación de la expresión genética de las porinas (De la Cruz and Calva, 2010).

9. PERSPECTIVAS

Este trabajo aportó un modelo de regulación novedoso en el área y detalló mecanismos moleculares que controlan la expresión de *ompS1*. Sin embargo, en este trabajo se generaron las siguientes perspectivas:

- Con respecto al papel de la curvatura intrínseca sobre la regulación de *ompS1*, evaluar el papel de curvaturas intrínsecas localizadas hacia el 5' que presentaron valores mucho más altos que la analizada en el presente estudio.

- Analizar el papel de ambos promotores de *ompS1*, evaluando condiciones ambientales que favorezcan la expresión de P1 y de P2, como osmolaridad, pH, tensión de oxígeno, medio de cultivo, analizando asimismo la estabilidad de cada mRNA.

- Mutagenizar cromosomalmente las cajas -10 ó -35 que inhiban la expresión de P1 y no de P2, y viceversa; evaluando estas construcciones en el modelo del ratón a fin de determinar el papel *in vivo* de P1 y de P2.

- Evaluar el papel del superenrrollamiento negativo del DNA mediado por las proteínas Fis y Topoisomerasa II.

10. REFERENCIAS

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The Complexities of Porin Genetic Regulation

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Key Words
Porin · *Escherichia coli* · *Salmonella enterica*

Abstract
Our understanding of porin regulation has revealed not only increasing complexity in the regulatory mechanisms, but also that the porin repertoire is more extensive than previously conceived. Initially, the OmpR response regulator was described as the master regulator of porin genes, but many more regulators are involved such as CpxR, PhoB, Lrp, Rob, MarA, SoxS, CadC, CRP, Fnr, ToxR, H-NS, StpA, IHF, HU and LeuO. In addition to MicF, the first small RNA (sRNA) that was proposed to regulate porin expression, porins are post-transcriptionally regulated by a variety of other sRNAs, namely, MicC, MicA, IpeX, RseX, InvR, CyaR and RybB. Future challenges include the full integration of all the regulatory circuitries. Whether quiescent porins are merely replacements of the major porins or are part of novel metabolic programs has yet to be elucidated. The comprehensive exploration of the environmental determinants that affect porin gene expression should yield valuable new information about the functions of these important proteins.

Porins: Gatekeepers of the Cell

The outer membrane is the hallmark of Gram-negative bacteria. It surrounds the cytoplasmic membrane and the peptidoglycan layer, and one of its more important functions is that of a permeability barrier. Porins are outer membrane proteins (OMPs) that produce large, open but regulated, water-filled pores that form substrate-specific, ion-selective or nonspecific channels that allow the influx of small hydrophilic nutrient molecules and the efflux of waste products. They also exclude many antibiotics and inhibitors (e.g. bile salts) that are large and lipophilic [Nikaido, 2003]. Much progress has been made in recent years towards the better comprehension of the complex genetic regulation of porins, a complexity that reflects the ability of didermic (2-membrane) bacteria to adapt to a wide variety of ecological niches.

Porins of the general bacterial porin family (Transporter Classification No. 1.B.1), including OmpC and OmpF of *Escherichia coli* [Saier et al., 2006, 2009], form stable trimers with a slight preference for cations over anions. OmpF seems to have a slightly larger channel than OmpC. OmpC and OmpF are the 'constitutive' major porins. The related PhoE porin has a general preference for anions but is not phosphate-specific, although its production is induced under phosphate limitation [Nikaido, 2003]. *Salmonella enterica* has, in addition to OmpC and

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OmpF, two quiescent general bacterial porins, namely OmpS1 and OmpS2, which are subject to complex regulatory mechanisms [De la Cruz et al., 2007]. The homologues of the *ompS1* and *ompS2* genes in *E. coli* are *yedS* and *ompN*, respectively [Fernández-Mora et al., 1995, 2004]. In this respect, in *E. coli* and in other Enterobacteriaceae, OmpG is a quiescent porin involved in the import and metabolism of oligosaccharides [Fajardo et al., 1998]. *S. enterica* serovar Typhimurium contains a characteristic abundant porin, OmpD, another member of the general bacterial porin family. In addition, there are other smaller monomeric porins such as OmpA, OmpX and OmpW [Nikaido, 2003; and see TCDB; www.tcdb.org].

In terms of virulence, abolition of porin production diminishes pathogenesis, both in the early stages of the infection (interaction with epithelial cells) and in later stages (interaction with macrophages). In *S. enterica* serovar Typhimurium, a double *ompC ompF* mutant, and single and double mutants in the quiescent genes *ompS1* and *ompS2* are attenuated for virulence in Balb/c mice [Chatfield et al., 1991; Rodríguez-Morales et al., 2006]. Interestingly, attenuation is seen upon oral inoculation, suggesting that OmpS1 and OmpS2 play traditional roles (allowing influx of nutrients, but not bile salts) in the gastrointestinal tract [Rodríguez-Morales et al., 2006]. In *Shigella flexneri*, OmpC participates in the interaction with macrophages [Bernardini et al., 1993]. An *ompX* mutant in *Yersinia pestis* showed diminished adherence and internalization when added to cultured HEp-2 cell monolayers, and was more susceptible to the bactericidal effect of human serum. The deletion of *ompX* resulted in a significantly reduced autoaggregation phenotype and loss of pellicle formation in vitro [Kolodziejek et al., 2007].

In terms of the responses to stresses in *E. coli*, mutants deficient in the OmpR porin regulator grew more slowly than the parental strain at low osmolarity and acid pH. Moreover, an *ompR* mutant is affected in the level of expression of many genes [Oshima et al., 2002]. Similarly, mutants in the *ompC* and *ompF* genes were also growth-deficient at high osmolarity and alkaline pH [Kaeriyama et al., 2006; Sato et al., 2000]. The *ompA* gene is repressed in acid pH [Huang et al., 2007]. The OmpD and OmpW porins in *S.* Typhimurium are reported to be involved in the detoxification of cells by means of the efflux of toxic compounds such as methyl viologen [Gil et al., 2007; Santiviago et al., 2002], which causes oxidative stress.

OmpR, Master Regulator of Porins

OmpR is the cytoplasmic response regulator of a two-component system (TCS), which also includes EnvZ, an inner membrane sensor histidine kinase. OmpR is a pleiotropic regulator that controls the expression of porin genes and cellular processes such as chemotaxis and virulence [Brzostek et al., 2007; Chatfield et al., 1991; Garmendia et al., 2003; Lee et al., 2000; Park and Forst, 2006; Shin and Park, 1995; Slauch and Silhavy, 1989]. The OmpR protein contains two domains: the N-terminal domain contains the phosphorylation site (D55) and the C-terminal domain contains the DNA-binding function in a winged helix-turn-helix motif [Kato et al., 1989]. The OmpR phosphorylation status is crucial for porin gene regulation. EnvZ is the main kinase that phosphorylates OmpR to form phosphorylated OmpR (OmpR-P). The environmental signals described so far that influence EnvZ activity are osmolarity, pH, temperature, nutrients and toxins [Forst and Inouye, 1988; Liu and Ferenci, 2001; Pratt et al., 1996]. However, under certain conditions, acetyl phosphate can function as a phosphate donor for OmpR [Bang et al., 2000; Hsing and Silhavy, 1997; Russo and Silhavy, 1991]. In addition to its kinase activity, EnvZ also acts as a phosphatase, controlling the concentration of OmpR-P in the cell [Qin et al., 2001]. OmpR-P is the active form of OmpR since phosphorylation increases its binding affinity for the promoter regions of the *ompC* and *ompF* genes [Forst et al., 1989; Head et al., 1998; Huang et al., 1997]. Interestingly, in addition to its activator role, OmpR-P is a repressor of certain genes [Brzostek et al., 2007; Shin and Park, 1995]. OmpR acts as a dimer, oligomerizing via β-sheets and binding to DNA in a head-to-head orientation [Maris et al., 2005]. The consensus OmpR-binding site consists of 20 base pairs (bp), providing a tandem site for two OmpR-P molecules [Harlocker et al., 1995; Harrison-McMonagle et al., 1999; Yoshida et al., 2006].

In *E. coli* K-12 at low osmolarity, the concentration of OmpR-P is low, but enough to interact with the high-affinity sites (F1-F2-F3) in the *ompF* regulatory region that are responsible for activating *ompF* transcription. At high osmolarity, the concentration of OmpR-P is much greater, allowing OmpR-P to occupy the sites in the *ompC* regulatory region responsible for activating *ompC* transcription, and the sites at *ompF* (F4) responsible for repressing *ompF* transcription [Pratt et al., 1996]. Thus, this model predicts that the regulation of *ompF* and *ompC* is a direct consequence of the level of OmpR-P in the cell and is dependent on the way in which OmpR-P interacts with sites in the *ompF* and *ompC* regulatory regions. A recent paper

described a novel mode of OmpR-P binding to the *ompC* and *ompF* regulatory regions, the so-called 'galloping mode'. This mechanism implies a tight hierarchical binding of OmpR, thus allowing a distinct stepwise regulation of *ompF* and *ompC* transcription, which minimizes their overlapping expression upon changes in the medium osmolarity to achieve the appropriate expression of *ompF* and *ompC* [Yoshida et al., 2006]. Interestingly, though, this model of reciprocal expression does not apply to *S. enterica* serovar Typhi and to *S. flexneri*, where OmpC production is constitutive [Bernardini et al., 1993; Puente et al., 1991]. Constitutive expression of either *S.* Typhi or *E. coli ompC* in an *S.* Typhi background is dependent on the presence of *S.* Typhi EnvZ [Martínez-Flores et al., 1999]. Induction by high osmolarity has been observed for *ompX* in *Enterobacter aerogenes*, where two putative OmpR-binding boxes have been tentatively identified [Dupont et al., 2004].

In addition, OmpR regulates the production of the OmpS1 and OmpS2 quiescent porins [Fernández-Mora et al., 2004; Oropeza et al., 1999]. Interestingly, OmpR differentially regulates *ompS1* promoter use by activating the P1 promoter and repressing the P2 promoter [De la Cruz et al., 2007; Flores-Valdez et al., 2003; Oropeza et al., 1999]. Moreover, the status of OmpR phosphorylation causes this differential regulation: OmpR-P activates P1 and represses P2, whereas P2 is active in the absence of OmpR-P [De la Cruz et al., unpubl. data].

Beyond OmpR: Other Global Regulators Involved in Porin Regulation

Other regulators of porin gene expression belong to several protein families, such as CpxR (which responds to extracytoplasmic stress) [Batchelor et al., 2005], PhoB (phosphate limitation) [von Krüger et al., 2006], Lrp (starvation) [Ferrario et al., 1995], Rob (cationic peptides), MarA (weak acids), SoxS (redox system) [Delihas and Forst, 2001], CadC (low pH) [Lee et al., 2007], CRP (catabolite repression), Fnr (anaerobiosis) [Santiviago et al., 2003], ToxR (virulence) [Miller and Mekalanos, 1988], H-NS, StpA, IHF, HU (nucleoid proteins) [Deighan et al., 2000; Painbeni et al., 1997; Ramani et al., 1992] and LeuO (stringent response) [Fernández-Mora et al., 2004; De la Cruz et al., 2007], among others (fig. 1).

CpxA-CpxR

CpxA-CpxR forms a TCS in which CpxA is the transmembranal sensor kinase and CpxR is the response regulator. CpxA can be induced by a variety of stimuli such as alkaline pH, misfolded proteins and alterations in the membrane composition, such as those related to pili and adhesins, in contrast to the misfolded OMPs which act as stimuli for Sigma E activation [Dorel et al., 2006; Raivio, 2005]. In addition, CpxP is a periplasmic protease that degrades CpxA and is necessary for the control of this TCS; especially upon activation of its expression by CpxR [Danese and Silhavy, 1998; Isaac et al., 2005]. Upon activation of the kinase activity of CpxA, the phosphorylated CpxR (CpxR-P) that is formed functions as a transcriptional activator or repressor of gene expression. CpxR-P positively and negatively regulates *ompC* and *ompF,* respectively, by binding to the promoter regions at sites that are both distinct from and overlap those for OmpR-P [Batchelor et al., 2005]. Interestingly, CpxR positively regulates the expression of *ompS1*, in addition to OmpR [De la Cruz et al., unpubl. data]. In addition to the 'classic' porin genes, OmpR and CpxR co-regulate several other genes including *nanC* [Condemine et al., 2005], *csgD* [Jubelin et al., 2005] and *inv* [Brzostek et al., 2007; Carlsson et al., 2007].

PhoR-PhoB

PhoR-PhoB is a TCS that senses the environmental phosphate concentration and, thus, responds to phosphate starvation. The PhoR sensor kinase detects a low concentration of environmental phosphate and activates PhoB, the response regulator by phosphorylation (PhoB-P) [Wanner, 1996]. One of the first proteins whose synthesis was reported to be affected by PhoB was the PhoE porin, which is produced under phosphate limitation [Tommassen and Lugtenberg, 1981]. PhoB-P directly activates the transcription of the *phoE* gene by binding to two Pho-boxes [Scholten and Tommassen, 1994]. In addition to PhoE, PhoB negatively regulates the OmpT, OmpU and OmpA major porins in *Vibrio cholerae* [von Krüger et al., 2006].

Lrp

Lrp is a global regulator that is widely distributed among bacteria and archaea, where it mainly regulates processes related to amino acid metabolism. It is often grouped together with the histone-like proteins due to its abundance in the cell (3,200 molecules per cell), its pleiotropic effects (affecting expression of 10% of *E. coli* genes) and its role in structuring the bacterial chromosome [Azam et al., 1999; Dame, 2005; Tani et al., 2002]. Lrp activity is stimulated in minimal medium, which reflects starvation and low nutrient availability, and repressed in

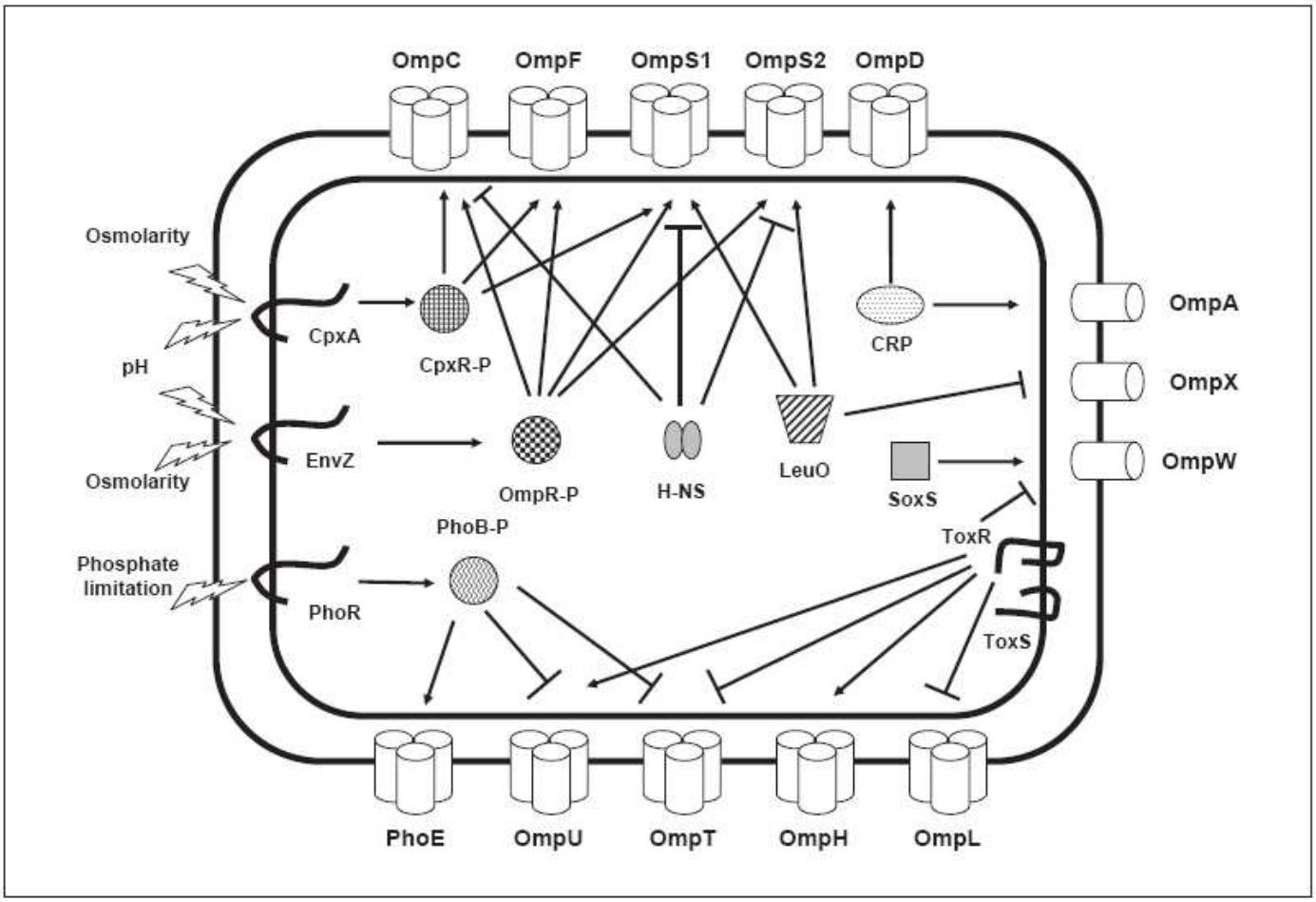

Fig. 1. The complexities of porin genetic regulation. Some aspects of the emerging regulatory network for bacterial porin regulation are shown, illustrating the variety of cytoplasmic and inner membrane-bound regulators and the porin repertoire in the outer membrane, including trimeric and monomeric species. OmpU and OmpT are from *V. cholerae*; OmpH and OmpL are from *Photobacterium*; OmpD is characteristic of *S.* Typhimurium; OmpS1 is distinctive of *S. enterica* as the equivalent gene in *E. coli (yedS)* has a truncated open reading frame; OmpW has been identified in *S. enterica*, *E. coli* and *V. cholerae*; and the rest are shared between *E. coli* and *S. enterica*. The master porin regulator is shown in its phosphorylated form (OmpR-P) interacting with the EnvZ sensor protein of this TCS, which senses environmental signals (e.g. osmolarity and pH). Similarly, the CpxR and PhoB regulators are depicted, as well as ToxR/ToxS, SoxS and CRP. The H-NS nucleoid protein is shown as a negative regulator, antagonized by LeuO for expression of OmpS1 and OmpS2. Details are described throughout the text.

rich medium [Calvo and Matthews, 1994]. In minimal medium, Lrp negatively and positively regulates *ompC* and *ompF*, respectively. Lrp represses *ompC* transcriptionally by binding to the *ompC-micF* intergenic region, which overlaps the promoter region; and it positively regulates *ompF* at the post-transcriptional level by repressing *micF*, which codes for MicF, a small antisense RNA that inhibits the translation of *ompF* mRNA [see below; Ferrario et al., 1995].

Rob, MarA and SoxS

MarA, SoxS and Rob are members of the AraC/XylS family of transcriptional regulators [Gallegos et al., 1997]. These three proteins diminish OmpF expression by activating *micF* transcription in response to different environmental stimuli [Delihas and Forst, 2001; Miller and Sulavik, 1996]. SoxR is a cytoplasmic sensor protein activated by oxidative stress that, in turn, transcriptionally activates the SoxS regulator [Li and Demple, 1994]. MarA responds to weak acids such as salicylic acid and aryloxoalcanoic acids [Balagué and Véscovi, 2001; Cohen et

al., 1993] and certain antibiotics [Hächler et al., 1991]; it positively regulates *ompX* in *E. aerogenes* [Dupont et al., 2004]. Rob may be a general transcriptional regulator, whose activity is enhanced by the binding of several effectors to its C-terminal region [Bennik et al., 2000; Rosenberg et al., 2003; Rosner et al., 2001]. Rob might be stimulated by cationic peptides since activation of *micF* under this condition is lost in a *rob* mutant, but not in *soxS* or *marA* backgrounds [Oh et al., 2000]. In addition, SoxS positively regulates the *ompW* porin gene in the presence of methyl viologen, in line with the identification of SoxS-binding sites in the regulatory region [Gil et al., 2009].

CadC

CadC is an inner membrane transcriptional activator that acts both as a signal sensor and as a transcriptional regulator, responding to low pH and lysine levels by activating transcription of the *cadBA* operon [Kuper and Jung, 2005; Rhee et al., 2005; Watson et al., 1992]. Using a proteomic approach, 8 of the putative CadC-induced proteins and 15 of the putative CadC-repressed proteins were identified where CadC positively regulated the production of OmpC and OmpF during acid adaptation. Interestingly, and contrary to what would be expected, the expression of *ompR* increased in a *cadC* mutant, illustrating the need for a better understanding of the control of OmpC and OmpF by CadC [Lee et al., 2007].

CRP

CRP is a master global regulator that controls a variety of carbon metabolic genes in bacteria. In *E. coli*, CRP affects transcription of more genes than any other regulator [Martínez-Antonio and Collado-Vides, 2003]. CRP positively and negatively regulates the expression of the *ompR-envZ* operon by binding directly to the promoter region [Huang et al., 1992]. In addition, in *S.* Typhimurium, transcription from the *ompD* porin gene promoter is activated by cAMP-CRP. The consensus CRP site, located 160–139 bp upstream of the *ompD* ATG start codon, might be the site required for the positive regulation by CRP [Santiviago et al., 2003]. The expression of the *E. coli ompA* gene is positively regulated by CRP, although the detailed mechanism has not been elucidated [Gibert and Barbé, 1990]. Recently, it has been reported that CRP negatively regulates *ompX* expression by means of CyaR, a new small RNA (sRNA) [see below; Papenfort et al., 2008].

Fnr

Fnr is a DNA-binding protein that senses changes in oxygen concentration and controls the expression of the different genes either alone or in cooperation with other regulators, such as ArcA [e.g. Chattopadhyay et al., 1997]. Fnr positively regulates the expression of *ompD* under anaerobiosis by a post-transcriptional regulatory mechanism that probably acts through mRNA stability [Santiviago et al., 2003].

ToxR

ToxR, a transmembrane DNA-binding protein that is homologous to OmpR, is an important regulator of virulence gene expression in *V. cholerae*. In conjunction with another membrane protein, ToxS, ToxR controls the expression of at least 17 genes in what is termed the ToxR regulon [Miller and Mekalanos, 1988; Peterson and Mekalanos, 1988]. ToxR positively regulates the expression of the *ompU* porin gene by binding directly to its regulatory region [Crawford et al., 1998]. In addition to *ompU*, ToxR negatively regulates the expression of the *ompT* porin gene by overlapping the promoter region [Li et al., 2000]. Interestingly, increased osmolarity enhances OmpT production and diminishes OmpU production [Miller and Mekalanos, 1988]. This reciprocal regulatory mechanism is similar to that of OmpR on *ompC* and *ompF* in *E. coli,* except that ToxR does not contain a phosphoacceptor domain. *ompW* is downregulated at high osmolarity in *V. cholerae*, where ToxR negatively regulates *ompW* in the presence of glucose [Nandi et al., 2005]. Moreover, the deep-sea bacterium Photobacterium SS3 contains a *toxRS* operon that regulates the synthesis of its OmpH and OmpL porins, which are produced at high and low hydrostatic pressure, respectively [Welch and Bartlett, 1998].

Small Nucleoid Proteins

Bacteria possess proteins with functional similarity to eukaryotic histones. This equivalence is not based on amino acid sequence relatedness, but on DNA-binding ability, their common low molecular masses, copy number and electrostatic charge. By analogy, they might also contribute to the organization of the bacterial nucleoid. Several of these histone-like proteins influence gene expression and other DNA-based functions, such as recombination and replication [Dorman and Deighan, 2003; Saier, 2008]. H-NS, StpA, IHF and HU, all of which appear to affect the expression of several porin genes, belong to this group.

H-NS is a master global regulator that represses transcription of a significant portion of the genome in enterobacteria (5–12%) [Baños et al., 2008; Hommais et al., 2001; Lucchini et al., 2006; Navarre et al., 2006]. It con-

trols the expression of several porin genes, such as those encoding OmpC, OmpF, OmpS1 and OmpS2. In the case of the major porins, H-NS represses *ompC* and diminishes the production of OmpF by regulating the level of MicF; that is, in an *hns* background, the MicF levels increase [Suzuki et al., 1996]. Interestingly, the stability of MicF is StpA-dependent. StpA, a paralogue of H-NS, is an RNA chaperone [Deighan et al., 2000]. In addition to its role as a chaperone, StpA has been described as a molecular back-up that acts in the absence of H-NS, having both overlapping and distinct functions [Sondén and Uhlin, 1996; Zhang et al., 1996]. H-NS and StpA silence *ompS1* expression in *S. enterica* by direct binding to its regulatory region [De la Cruz et al., 2007; Flores-Valdez et al., 2003]. Interestingly, the DNA intrinsic curvature favors the repression of H-NS and StpA on *ompS1* [De la Cruz et al., 2009]. Similarly, H-NS negatively regulates *ompS2 (ompN)* expression in *E. coli* and *S.* Typhimurium [Hommais et al., 2001; Navarre et al., 2006]. On the other hand, H-NS and StpA stimulate the production of the outer membrane maltoporin LamB through post-transcriptional control of MalT, the maltose regulon activator [Johansson et al., 1998].

IHF, one of the most abundant sequence-specific DNA-binding proteins, was first identified and isolated as a host factor for integrative recombination of phage λ [Craig and Nash, 1984; Nash and Robertson, 1981]. IHF is now recognized as a global regulator [Ishihama, 1997]. The native form of IHF is a heterodimer with subunits of similar amino acid sequence [Nash and Robertson, 1981]. The IHF protein negatively regulates *ompC* expression by binding directly to the regulatory region, protecting a 35-bp region located upstream from the *ompC* promoters [Huang et al., 1990]. IHF which is necessary for the negative osmoregulation of *ompF* binds to the promoter region and structures the DNA [Ramani et al., 1992; Tsui et al., 1988]. In addition to *ompC* and *ompF*, IHF directly and negatively regulates the *ompR-envZ* operon [Tsui et al., 1991]. Thus, IHF affects *ompC* and *ompF* in two distinct ways: directly by binding upstream to the promoter regions, and indirectly by influencing the expression of OmpR and EnvZ [Huang et al., 1990].

HU is a nucleoid protein that is more analogous in function to the eukaryotic HMG proteins [Oberto et al., 1994]. The HU protein is composed of two closely related subunits, the HUβ subunit, encoded by *hupB* gene, and the HUα subunit, encoded by the *hupA* gene [Kano et al., 1986, 1987]. An *E. coli hupAB* mutant exhibits increased OmpF porin levels through diminished *micF* expression [Painbeni et al., 1997].

LeuO

LeuO is a LysR-type regulator that controls the expression of several genes implicated in the bacterial response to stress, in virulence and in biofilm accumulation [Lawley et al., 2006; Moorthy and Watnick, 2005; Rodríguez-Morales et al., 2006; Tenor et al., 2004]. Recently, proteomic analysis demonstrated that several genes are regulated by LeuO in *S.* Typhi [Hernández-Lucas et al., 2008]. The OmpS1 and OmpS2 quiescent porins are strongly silenced by H-NS [Flores-Valdez et al., 2003; Navarre et al., 2006]. LeuO acts as an antagonist of H-NS, thereby derepressing *ompS1* and *ompS2* expression [De la Cruz et al., 2007; Fernández-Mora et al., 2004]. The derepressing effect of LeuO is concentration-dependent: low levels of LeuO activate *ompS2* expression while *ompS1* is repressed, and a high level of LeuO represses *ompS2* and activates *ompS1* expression [De la Cruz et al., 2007]. The LeuO-binding sites at *ompS1* and *ompS2* are located upstream and do not overlap with the promoter regions, a unique characteristic of this regulator. Interestingly, upon derepression of LeuO, *ompS1* and *ompS2* are positively regulated at high osmolarity, similarly to the *E. coli ompC* gene [De la Cruz et al., unpubl.; Fernández-Mora et al., 2004]. Additionally to its role as an activator of gene expression, LeuO can also repress transcription, as in the case of the *ompX* porin gene in *S.* Typhi [Hernández-Lucas et al., 2008]. Thus, LeuO can positively and negatively modulate the porin repertoire.

Small RNAs: Adding to the Complexity

Small untranslated regulatory RNAs, usually referred to as noncoding RNAs, are present in all kingdoms of life [Argaman et al., 2001]. Moreover, the first RNA described with such properties was MicF, involved in porin regulation [Andersen et al., 1987; Mizuno et al., 1984]. Generally, they inhibit translation of the transcripts by direct RNA-RNA interaction. The sRNAs play diverse physiological roles in the response to stress, regulation of metabolism, control of bacterial envelope composition and bacterial virulence [Majdalani et al., 2005; Romby et al., 2006; Storz et al., 2005; Vogel and Papenfort, 2006]. Recent studies show that enterobacteria use many sRNAs to fine-tune the outer membrane composition at the post-transcriptional level, namely, MicC, MicA, InvR, RybB, CyaR, IpeX and RseX [Vogel and Papenfort, 2006] (fig. 2).

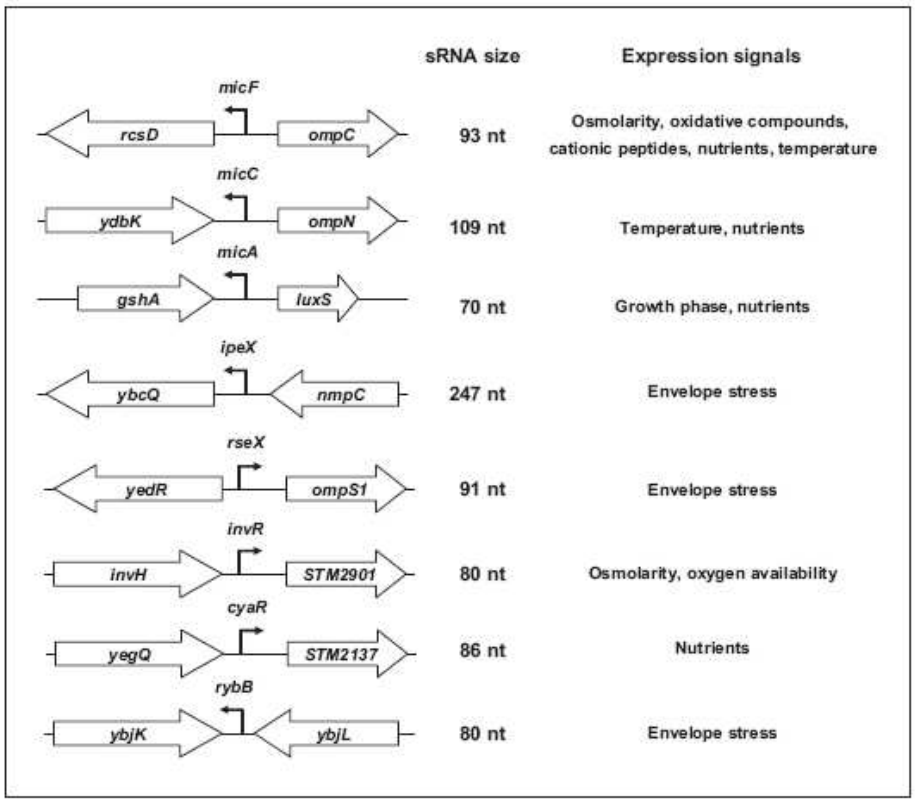

Fig. 2. Porin regulatory sRNAs. Several of the porin regulatory sRNA genes are adjacent to porin genes, such as *micF*, *micC*, *ipeX* and *rseX*, suggestive of a concerted porin regulation. Most of them share a similar size except for *ipeX*, which is proposed to form an extensive secondary structure [Castillo-Keller et al., 2006]. The environmental signals for sRNA expression are indicated. Details are described throughout the text.

MicF

E. coli MicF was discovered more than 20 years ago [Mizuno et al., 1984] and was the first sRNA characterized. The *micF* gene was identified through its ability to repress OmpF production when present in multiple copies. The 93-nt (nucleotide) MicF forms a 20-bp imperfect RNA duplex with the translation-initiation region of *ompF* mRNA [Schmidt et al., 1995] to regulate expression negatively. Environmental factors such as oxidative compounds, toxic compounds and nutrients activate *micF* expression, thereby decreasing OmpF levels [Delihas and Forst, 2001]. MicF levels change inversely with respect to the concentration of nutrients in the growth medium, i.e. MicF is low in minimal media but high in rich media [Coyer et al., 1990]. Activation of *micF* transcription occurs upon a temperature or osmolarity increase, or upon exposure to cationic peptides [Oh et al., 2000]. *micF* expression is highly regulated through a complex promoter region that is rich in binding sites for transcriptional regulators, including OmpR, Lrp, SoxS, Rob, MarA, and H-NS [Delihas and Forst, 2001]. Moreover, the post-transcriptional stability of MicF is determined by StpA, as described above.

MicC

MicC is an sRNA of 109 nt encoded in the *ompN (ompS2)-ydbK* intergenic region [Chen et al., 2002]. MicC negatively regulates *ompC* expression at the post-transcriptional level by base-pairing with *ompC* mRNA and preventing the formation of an active translation initiation complex. MicC is also bound by the Hfq RNA chaperone which is required for MicC regulation of OmpC levels [Chen et al., 2004]. Therefore, Hfq is a highly conserved pleiotropically acting RNA-binding protein involved in the post-transcriptional regulation by sRNAs of many stress-responsive genes. Thus, an increase in MicC production correlates with a decrease in MicF production. For example, the levels of MicC are elevated at low temperature and limitation of nutrients. OmpR appears to repress *micC*, either directly or indirectly [Chen et al., 2004].

MicA

MicA was initially found in a computational screen for sRNAs [Argaman et al., 2001]. It is abundant in the stationary phase in rich medium. The possibility that

Table 1. Genetic regulation of porin expression

Porin	Environmental signals	Regulator(s)	Indirect regulator(s)	sRNA(s)
OmpC	osmolarity, pH, temperature	OmpR, CpxR, Lrp, IHF, H-NS	CRP, CadC	MicC, IpeX, RseX, RybB
OmpF	osmolarity, temperature	OmpR, CpxR, IHF	CRP, CadC, HU, StpA, Rob, MarA, SoxS	MicF, IpeX, RybB
OmpA	pH		CRP, PhoB	MicA, RseX, RybB
OmpS1	osmolarity	OmpR, CpxR[1], LeuO, H-NS, StpA		RybB
OmpS2 (OmpN)	osmolarity	OmpR, LeuO, H-NS		RybB
PhoE	phosphate limitation	PhoB		
OmpX	osmolarity	LeuO, OmpR[1], MarA[1]	CRP	CyaR
OmpW	osmolarity, oxidative stress	SoxS	ToxR	RybB
OmpD (*S.* Typhimurium)	anaerobiosis	CRP[1]	Fnr	InvR, RybB
OmpU *(V. cholerae)*	osmolarity	ToxR	PhoB	
OmpT *(V. cholerae)*	osmolarity	ToxR	PhoB	
OmpH *(Photobacterium)*	hydrostatic pressure	ToxR		
OmpL *(Photobacterium)*	hydrostatic pressure	ToxR		
OmpL *(E. coli)*	redox potential			
Specific channel protein				
LamB	maltose	CRP, MalT	H-NS, StpA	MicA

[1] The DNA-protein interaction has not been experimentally evaluated, but consensus sequences were found.

MicA might regulate *ompA* expression was suggested by the complementarity between MicA and the 5′ sequence of the *ompA* mRNA, as well as by the observation that the overproduction of MicA leads to reduced OmpA protein levels [Rasmussen et al., 2005; Udekwu et al., 2005]. This control requires Hfq, which facilitates the MicA-*ompA* interaction, at least in part, by binding both the *ompA* mRNA and MicA. Consistent with its expression pattern, MicA accounts for the destabilization of the *ompA* transcript in the stationary phase in vivo; under these conditions, OmpA levels are significantly elevated in a strain lacking MicA. In addition to OmpA, MicA also downregulates the LamB maltodextrin porin gene in *S.* Typhimurium. In strains overexpressing σ^E, MicA accumulation leads to a significant decrease in LamB protein and mRNA levels. LamB downregulation by MicA requires a functional Hfq protein [Bossi and Figueroa-Bossi, 2007].

InvR

InvR (invasion gene-associated RNA) is encoded in the *Salmonella* pathogenicity island 1 (SPI-1). This abundant sRNA was identified in a global search for new *Salmonella* sRNA genes. InvR is activated by the major SPI-1 transcription factor HilD under conditions that favor host cell invasion (high osmolarity and low oxygen). Hfq is essential for the in vivo stability of the ca. 80-nt InvR. InvR represses the synthesis of the abundant OmpD porin encoded by the *S.* Typhimurium core genome at the post-transcriptional level. As InvR is conserved in *Salmonella bongori*, which branched early from *S. enterica* and contains SPI-1, it has been speculated that porin gene repression by InvR has aided the successful establishment of such a pathogenicity island after horizontal acquisition in the *Salmonella* lineage [Pfeiffer et al., 2007].

RybB

RybB is an sRNA of 80 nt regulated by σ^E that was initially identified in a computational screen for conserved intergenic sequences [Vogel et al., 2003; Wassarman et al., 2001]. RybB targets a large set of porin mRNAs including *ompA*, *ompC*, *ompD*, *ompF*, *ompN (ompS2)*, *ompS1*, *ompW* and *ompX*. RybB facilitates *omp* mRNA decay as part of

the envelope stress response: by direct interaction with their 5′ untranslated regions in an Hfq-dependent fashion [Papenfort et al., 2006].

CyaR

CyaR is a new Hfq-dependent sRNA that acts as an *ompX* repressor by sequestering the Shine-Dalgarno sequence of *ompX* mRNA and inhibiting translational initiation. The expression of *cyaR* is tightly controlled by the cyclic AMP receptor protein, CRP. This represents a new link between porin gene repression and nutrient availability that is likely to be widely conserved among enterobacteria [Papenfort et al., 2008].

IpeX and RseX

IpeX (inhibitor of porin expression) and RseX (RNA suppressor of extracytoplasmic stress protease) have been identified as multicopy suppressors. IpeX is encoded in the *ybcQ-nmpC* intergenic region [Douchin et al., 2006; Pugsley and Schnaitman, 1978]. IpeX down-regulates both OmpC and OmpF: IpeX-mediated destabilization of the *ompC* mRNA is independent of Hfq [Castillo-Keller et al., 2006].

RseX is a 91-nt RNA encoded on the same strand and upstream of *yedS*, which encodes a putative homologue of OmpS1 [Fernández-Mora et al., 1995]. The *ompA* and *ompC* transcripts were identified as targets of RseX [Douchin et al., 2006].

Corollary

It is noteworthy that not only our understanding of porin regulation has revealed an increasing complexity in the regulatory mechanisms, but that the porin repertoire, itself, is more extensive than previously conceived (table 1). In particular, our knowledge of the quiescent porins is at its initial stages. Porins in this category include OmpS1, OmpS2 (OmpN), NmpC, Lc, OmpG and OmpL *(E. coli)*. NmpC was discovered as a suppressor of major porin-less strains and is considered as the product of a 'silent' gene for an OMP that would serve as a replacement of the major porins [Pugsley and Schnaitman, 1978]. Similarly, OmpG was discovered among mutants that increased outer membrane permeability in the absence of OmpF and LamB [Misra and Benson, 1989]. Therefore, elucidation of whether quiescent porins are merely replacements of the major porins or act in response to particular stress conditions that cannot be reproduced easily in the laboratory should greatly enhance our understanding of the function of the outer membrane. If they have characteristic roles, one can envision the quiescent porins as part of novel metabolic programs that are yet to be discovered.

The complexity of the *ompC ompF* reciprocal regulation in *E. coli* upon a shift in osmolarity [Pratt et al., 1996; Yoshida et al., 2006] is also intriguing if the major porins are considered to have a nonselective nature, and implies that they have distinct functional roles in specific environmental niches.

Genetic regulation of porin expression clearly entails complex transcriptional and post-transcriptional events. For instance, OmpS1 is subject to positive and negative transcriptional regulatory mechanisms involving OmpR, CpxR and LeuO, and H-NS and StpA, respectively [De la Cruz et al., 2007; unpubl. observ.]. Yet an unknown environmental signal or signals might derepress the expression of *leuO*, which in response turns on a specific set of genes by antagonizing H-NS action at a subset of regulatory sites. In this respect, a formidable challenge is the comprehensive exploration of the environmental determinants that affect the activity of the transcriptional regulators and the mechanistic details. At the same time, we will need to integrate the intricacies of the emerging knowledge on post-transcriptional regulation by sRNAs, which appear as fine modulators of porin composition.

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