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CENTRO DE CIENCIAS GENÓMICAS
DOCTORADO EN CIENCIAS BIOMÉDICAS

LA TRANSFERENCIA CONJUGATIVA DEL PLÁSMIDO SIMBIÓTICO DE *Rhizobium etli* ES MODULADA POR MEDIO DE INTERFERENCIA TRANSCRIPCIONAL Y REPRESIÓN

TESIS

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS

PRESENTA

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RESUMEN

Rhizobium etli es una bacteria gram negativa que posee seis plásmidos. El pSym, p42d, es un plásmido de 371 kb que es movilizado por cointegración con el p42a, por medio de un sistema de transporte *tra/trb* codificado en este último. En ausencia del plásmido p42a no se había detectado movilización del plásmido simbiótico en laboratorio. A pesar de esto, la secuencia del genoma de *R. etli* mostró la existencia de un sistema de transporte tipo *tra/vir* en el plásmido simbiótico. El sistema Dtr está compuesto de 4 genes *tra* mientras que el de Mpf está codificado en un operón *virB1-11*. Anteriormente, nuestros colaboradores habían aislado mutantes capaces de movilizar el pSym a frecuencias altas en ausencia de p42a. En este trabajo, al analizar estas mutantes por medio de fusiones transcripcionales**,** hemos logrado identificar dos genes involucrados en la regulación de este sistema. *rctA* es un gen situado cerca del operón virB, y funciona como un represor del sistema conjugativo del pSym; a través de predicciones computacionales se determinó que su producto, RctA, posee un dominio de unión a DNA del tipo hélice alada. También identificamos otro gen, *rctB*, como un inhibidor de la actividad represora de RctA. Utilizando protecciones (footprinting) con DNasa I y ensayos de retardo, hemos demostrado que existe una unión específica de RctA con el promotor del operón *virB*. Así mismo, hemos determinado que un motivo de 9 pares de bases, localizado en la región espaciadora de este promotor, así como la presencia de una caja -10 funcional, son necesarios para que se lleve a cabo esta interacción. Nuestros análisis con fusiones transcripcionales revelaron que la eliminación de cualquiera de estos elementos abole la represión ejercida por RctA. Estos datos apoyan un modelo en el que RctA bloquea el acceso de la RNA polimerasa al promotor del operón *virB*. Curiosamente, hemos encontrado que los niveles de expresión de rctA son modulados por medio de interferencia transcripcional emanada del promotor *virB*. Este fenómeno añade otro nivel de regulación para este sistema, lo que revela un nuevo mecanismo de regulación de la transferencia conjugativa en las Rhizobiales.

ABSTRACT

Rhizobium etli is a gram negative bacteria that contains six plasmids. The pSym, p42d, is a 371 kb plasmid that is mobilized by cointegration with p42a, using a *tra/trb* transfer system. No mobilization of pSym was found in the absence of p42a under laboratory conditions. Despite this, the complete sequence of *R. etli* showed the existence of a *tra/vir* system in p42d. The Dtr system is composed of 4 *tra* genes while the Mpf system is encoded in a *virB1-11* operon. Previously, we had obtained mutants that mobilized pSym at high frequencies in the absence of p42a. Using transciptional fusions, we identified two genes as important regulators of this system. *rctA* is a gene located near the *virB* operon that is a repressor of the pSym conjugal system. As a good candidate for a transcriptional regulator, RctA is predicted to have a DNA-binding winged helix domain. We also identified another gene, *rctB*, as an inhibitor of the repressor activity of RctA. Using DNase I footprinting and binding assays, we demonstrated the specific binding of RctA to the virB operon promoter. A 9-bp motif in the spacer region of this promoter (the rctA binding motif box) and the presence of a functional -10 region, were critical elements for RctA binding. Transcriptional fusion analyses revealed that the elimination of either element provoked a relief of RctA- mediated repression. These data support a model in which RctA inhibits the access of the RNA polymerase to the virB promoter. Interestingly, rctA expression levels were modulated by transcriptional interference from transcripts emanating from the virB promoter. This phenomenon adds another level of regulation for this system, thus revealing a novel mechanism of plasmid transfer regulation in the Rhizobiales.

INTRODUCCIÓN

Conjugación Bacteriana

La conjugación es un proceso, dependiente de contacto celular, por el que una molécula de DNA es transferida de una célula donadora a una célula receptora. Este fenómeno fue descrito en la década de los 40s´ por Joshua Lederberg y Edward Tatum a partir de la conjugación mediada por el plásmido F en *Escherichia coli* y desde entonces se ha descrito su ocurrencia entre una amplia gama de microorganismos. Por ejemplo, en condiciones naturales existe evidencia de conjugación entre las bacterias que habitan en los tractos digestivos del ser humano (*E. coli*, *Shigella flexneri*, *Salmonella enteriditis*, *Enterococus faecalis*, *Bifidobacterium sp.*, etc), en tejidos vegetales (*P. syringae*, *Pseudomona fluorescens*, *P. putida*, etc.) y en tractos digestivos de larvas y adultos de insectos (*Bacillus thuringiensis*, *E. cloacae*, *Erwinia herbicola*) (Davison, 1999). También se ha reportado conjugación entre bacterias gram positivas de los géneros *Streptomyces* y *Mycobacterium* (Grohmann, et al., 2003). Este fenómeno tiene gran relevancia biológica ya que es responsable de la propagación de características genéticas que son importantes para la adecuación de los organismos (Schröder *et al.,* 2002; Dobrindt y Reidl, 2000) y que se encuentran comúnmente codificadas en plásmidos (Kado, 1998), en transposones conjugativos y en islas genómicas (Zechner *et al.,* 1998). Por ejemplo, el plásmido RP4 de *P. aeruginosa*, el R26 de *Serratia marcescens* y el R6K de *E. coli* median la dispersión en las poblaciones bacterianas de la resistencia a tetraciclina, estreptomicina y penicilina, respectivamente. La movilización del plásmido lineal SCP1 de *S. coelicolor* dispersa vías de síntesis de antibióticos y la conjugación de los plásmidos pK88 de *E. coli*, pTi de *Agrobacterium tumefaciens* y pX02 de *Bacillus anthracis* distribuyen caracteres asociados a la patogenicidad (Top *et al.*, 2000).

En bacterias Gram-negativas la transferencia conjugativa se lleva a cabo a través de los sistemas de secreción tipo IV. Estos transportadores se encuentran ampliamente distribuidos y cumplen diversas funciones en los organismos que los poseen. Por ejemplo, en el caso de los transportadores

de las bacterias patógenas *Bordetella pertussis* (Burns, 1999), *Helicobacter pylori*, *Shigella* y *Neisseria,* éstos son utilizados únicamente para el envío de factores de patogenicidad hacia las células de su huésped (Lai y Kado, 2000) mientras que otros, como los codificados por los plásmidos de enterobacterias, se ocupan exclusivamente de la transferencia conjugativa de éstos (Zechner *et al.,* 1998). Un caso particular es el de la bacteria patógena de plantas *Agrobacterium tumefaciens*, cuyo plásmido Ti codifica dos diferentes sistemas tipo IV. Uno de ellos es responsable de la transferencia del plásmido Ti entre bacterias (sistema *tra-trb*), mientras que el otro (sistema *vir*) moviliza únicamente un fragmento de DNA del plásmido (denominado T-DNA) hacia las células vegetales, lo que deriva en la formación de tumores en las raíces (Hamilton *et al.,* 2000).

Los genes que integran los sistemas de excreción tipo IV suelen encontrarse organizados en operones y se dividen en dos clases: los requeridos para realizar el acoplamiento entre bacterias (Mpf) y los necesarios para el procesamiento del DNA (Dtr) (Baron *et al.,* 2002).

El sistema de procesamiento de DNA (Dtr)

Este sistema se encarga de convertir el DNA transferible en un sustrato movilizable por el sistema Mpf y está constituido por una proteína llamada relaxasa y otras proteínas accesorias. La relaxasa es una proteína con actividad de helicasa y de nucleasa, que es capaz de reconocer y de unirse a una secuencia conservada que poseen todos los plásmidos movilizables y que se denomina origen de transferencia (*oriT*) (Zechner *et al.,* 1998). Las proteínas accesorias interactúan con la relaxasa en regiones adyacentes al *oriT* para facilitar el reconocimiento y el procesamiento de éste, formando un complejo multi-proteico denominado relaxosoma. Aunque las proteínas accesorias no son esenciales para el proceso sí juegan un papel importante, ya que mutaciones en estas provocan una disminución en la tasa de transferencia (Farrand, 1996).

Una vez que el relaxosoma se ha unido al origen de transferencia la tirosina catalítica de la relaxasa realiza un ataque nucleofílico contra el extremo 5´ de un sitio específico del *oriT* llamado sitio *nic*. Como resultado, la

relaxasa queda firmemente unida a éste por un enlace fosfotirosil, mientras que el extremo 3` queda libre (Schröder *et al.,* 2002). Posteriormente, un proceso de replicación de tipo círculo rodante permite la separación de la molécula de DNA linearizada, al tiempo que se restituye la condición de doble cadena del plásmido original. La relaxasa es transferida junto con el DNA a la célula receptora (Draper, 2005) donde cataliza la recircularización del DNA a través de una segunda reacción de transesterificación (Llosa *et al.,* 2002). Debido a que los aparatos conjugativos han sido descritos en diferentes plásmidos, los componentes del sistema de procesamiento de DNA reciben diferentes nombres dependiendo de su origen (*vir* en el pTi de *A. tumefaciens* y *tra* en los plásmidos IncF e IncP).

El Sistema de Formación del Par Conjugativo (Mpf)

Los genes del sistema Mpf están generalmente organizados en un operón, sus componentes están muy conservados en diferentes organismos; aunque existen algunas diferencias, éstas pueden atribuirse a razones funcionales. Debido a esto, los componentes del sistema de formación del par conjugativo reciben diferentes nombres dependiendo de su origen (*vir* en el pTi de *A. tumefaciens*, *tra* en los plásmidos IncF y *trb* en los plásmidos IncP) (Fig. 1):

VirB1.- es una mureinasa , que se cree facilita el ensamble de los componentes del aparato conjugativo (Lai y Kado, 2000).

VirB2 o pilina.- El pili, la estructura característica de los sistemas conjugativos. está formado por subunidades de esta proteína. Su principales funciones parecen ser el reconocimiento de la célula receptora y el acercamiento por retracción a ésta (Lai y Kado, 2000). Aunque se ha sugerido que éste puede ser también el conducto de transferencia del complejo DNA-relaxosoma y de las proteínas que se transfieren al huésped, no hay evidencia experimental que compruebe esta teoría (Christie y Vogel, 2000).

VirB3.- Es la proteína mas pequeña del operón (100 aa) y se encuentra frecuentemente asociada a membrana externa, también se sabe

que establece contactos con la NTPasa VirB4 y con VirB5 (Shamei-Tousi et al., 2004).

VirB4.- Es la proteína más grande del sistema (800 aa) que posee dominios Walker A y B, por lo que considera que es una NTPasa que energiza el sistema y contribuye con la transferencia de las proteínas estructurales del sistema. Debido a que también interactúa con la proteína acopladora, se cree que también participa en la transferencia de los sustratos transportados (Atmakuri y Christie, 2004).

VirB5.- Es una proteína de aproximadamente 220 aa que se localiza extracelularmente asociada al pili. Se considera que participa en la identificación y la adhesión del pili a la célula receptora; en algunos sistemas, es una proteína receptora de bacteriófagos (Dehio, 2004).

VirB6.- Es una proteína localizada en la membrana interna. Se requiere para la interacción del DNA con los componentes del sistema que están asociados a la membrana externa, así mismo participa en la elongación del pili, aunque no es necesaria para la iniciación del mismo (Jakubowski et al., 2003).

virB7.- Esta proteína se encuentra tanto en la cara periplásmica de la membrana externa como extracelularmente asociada al pili. Su función es estabilizar el pili y servir de interfase de éste con los componentes centrales del aparato de secreción por medio de *virB9* (Krall et al., 2002)

VirB8.- Se localiza en la membrana interna y el periplasma. Forma parte del complejo central del sistema y su papel es el de servir como centro de nucleación, reclutando a *VirB9* y *VirB10* para iniciar el ensamble del aparato conjugativo (Kumar et al., 2000)

VirB9.- Se localiza en la membrana externa y en el periplasma, forma parte del complejo central del sistema y se cree que auxilia en la estabilización del sistema vía interacciones con *VirB7* (Beaupré et al., 1997).

VirB10.- El complejo central del sistema excretor es el puente que cruza el espacio periplásmico, siendo *VirB8* el ancla a la membrana interna y *VirB9* la de la membrana externa. *VirB10* se encuentra asociada a ambas. Tiene además la capacidad de interactuar con las NTPasas *VirD4* y *VirB4* y presenta cambios conformacionales dependientes de ATP, de los que depende su interacción con *VirB9.* Debido a que recientemente se ha

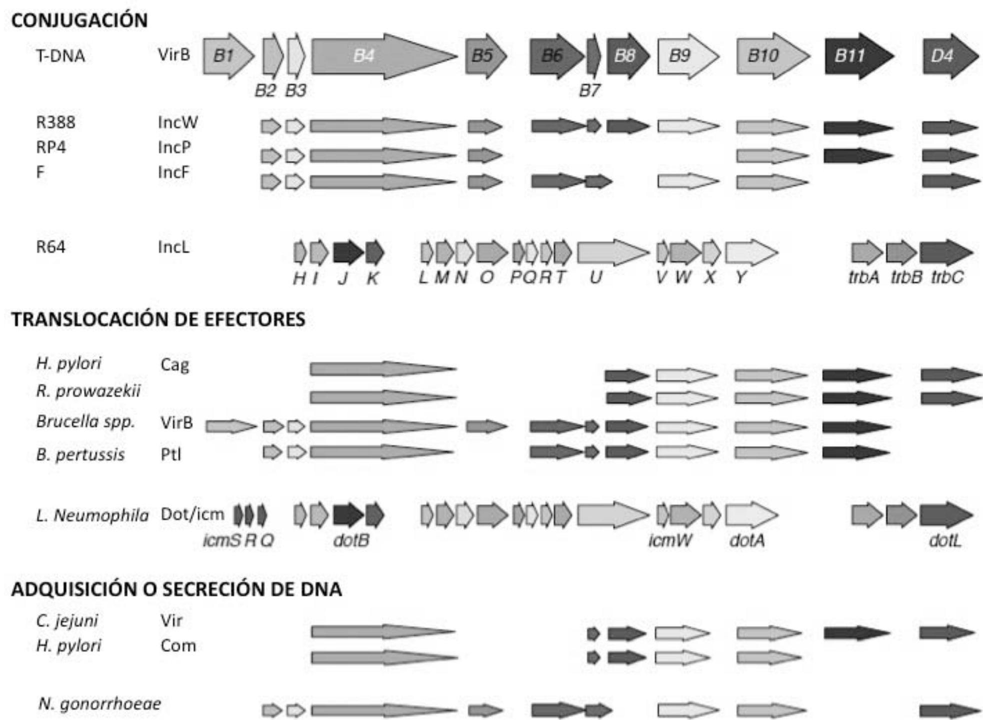
descubierto que la utilización de ATP por VirB4 la responsable de éstos cambios conformacionales, se propone que funciona como una compuerta del sistema central que sólo permite el paso de los sustratos transferibles, cuando éstos están siendo transportados por las NTPasas específicas de este (Cascales y Christie, 2004).

VirB11.- Pertenece a una familia de NTPasas que también se encuentran en los sistemas tipo II (Planet et al., 2001), se asocia a la membrana interna en forma de anillos hexaméricos. Debido a que interactúa con gran cantidad de componentes del sistema de secreción (VirB1, VirB4, VirB8, VirB9 y VirB10) y a que su sobre-expresión desestabiliza la membrana celular, se cree que se encarga del transporte de los componentes del núcleo al periplasma (Machón et al., 2002; Schröder y Lanka, 2003).

Como se mencionó anteriormente, los sistemas tipo IV presentan conservación en la secuencia y en el orden de los genes (fig. 1A) tanto en los de Dtr como en los de Mpf, por lo que la variedad en los substratos transportados sugiere una divergencia funcional a partir de un origen común (Cao y Saier, 2001). Inclusive, en el caso de los dos sistemas presentes en *A. tumefaciens* éstos son considerados híbridos, ya que en el sistema *tra* los genes de Dtr y el *oriT* están relacionados con los del plásmido RSF1010 y los de Mpf están relacionados con el plásmido RP4 (incP). Los genes de Mpf del sistema *vir* están relacionados con los de los plásmidos IncN; mientras que los genes de Dtr del mismo sistema están relacionados con los del plásmido RP4.

Recientemente se han encontrado algunos sistemas tipo IV, que transportan exclusivamente proteínas, en que la secreción de éstas depende de la presencia de una señal específica en su carboxilo terminal (Cascales y Christie, 2003). Esta señal también se ha identificado en efectores proteicos que son secretados por los sistemas VirB de *Bartonella henselae* y *A. tumefaciens* y se cree que se originó a partir de una relaxasa ancestral. El hecho de que existan sistemas tipo IV que secreten únicamente proteínas y de que en los que sí llevan a cabo transferencia de DNA ésta se realice acoplada a una proteína, hizo que se considerara que estos sistemas se habían originado como secretores de proteínas y que posteriormente se adaptaron para transferir DNA (Christie y Vogel, 2000).

A



B

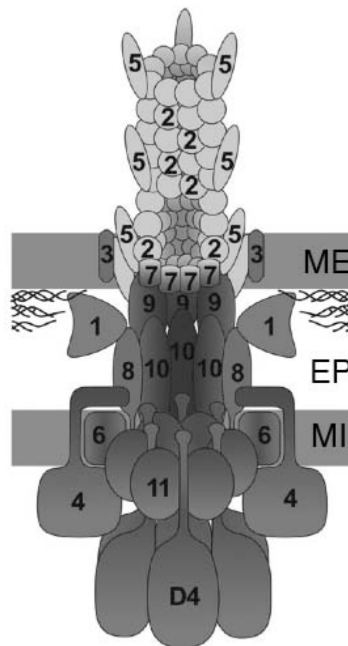


Figura 1.- A) Conservación de secuencia del operón vir en diferentes sistemas tipo IV según su función. B) Estructura del sistema de transporte tipo IV. ME.- Membrana Externa; EP.- Prisplasma; MI.- Membrana Interna. (Modificadas de Schröder y Lanka, 2005).

La Proteína Acopladora (TraG/VirD4)

El complejo relaxasa-DNA no es reconocido directamente por el aparato secretor; es necesaria la participación de una proteína acopladora entre el sistema de procesamiento de DNA y el de formación del par conjugativo. Esta proteína, que tiene actividad de NTPasa , está presente en todos los sistemas tipo cuatro. Se localiza en el citoplasma asociada a la membrana interna, en donde forma una estructura hexamérica en forma de anillo (Hormaeche *et al.*, 2002); aunque es esencial para la transferencia conjugativa de DNA, no lo es para el ensamble del pili o para la formación del par conjugativo (Lai y Kado, 2000). Bioquímicamente se ha demostrado que es capaz de establecer contactos con la relaxasa y con otros componentes del relaxosoma (Llosa *et al.*, 2003). También se asocia con los otros efectores secretados (Atmakuri *et al.*, 2003) y aunque también es capaz de unirse al DNA, esta interacción es inespecífica (Schröder y Lanka, 2003). De los componentes del aparato secretor, establece contactos con VirB10, VirB11 y VirB4, siendo la primera componente del núcleo del sistema y las dos últimas NTPasas (Malek et al., 2004).

Al ser la interfaz entre el sistema Mpf y el de Dtr, la proteína acopladora (PA) es la responsable de determinar la especificidad del sistema. Si una PA es capaz de reconocer el sistema Mpf de un plásmido y el Dtr de otro, entonces el primero podrá usar al segundo para movilizarse. Por ejemplo, los genes de Mpf del sistema Vir de *A. tumefaciens* son capaces de movilizar el plásmido RSF1010, que sólo posee genes de Dtr, ya que la PA tipo TraG de este último es capaz de acoplar ambos sistemas (Hamilton *et al.,* 2000).

El Mecanismo Conjugativo

A nivel celular, la conjugación inicia cuando una célula donadora extiende el pili en busca de establecer contacto con una célula receptora. Una vez que el contacto se ha realizado y si no existe incompatibilidad entre las células participantes, el pili sufre una retracción ocasionada probablemente por su depolimerización (Fig. 2). Una vez que el pili se ha

retraído, se forma un contacto íntimo entre la bacteria donadora y la receptora (en el que no se pueden distinguir dos membranas independientes) y durante el cual se lleva a cabo la transferencia de DNA.

A nivel molecular aún no está bien definido el mecanismo que permite la movilización del DNA. Ensayos bioquímicos de entrecruzamiento han permitido determinar que el primer contacto del complejo DNA-Relaxasa, tras pasar por la PA, es la NTPasa VirB11, la cual a continuación interactúa con la VirB6 y con la proteína del complejo central VirB8. Posteriormente hace contacto con VirB9, otro componente del complejo central, para finalmente interactuar con la pilina, VirB2 (Schröder y Lanka, 2003). Estos resultados, además de confirmar el rol primordial del complejo central en la transferencia evidencian que el pili también juega un papel importante en ésta. La idea de que el pili funciona como una jeringa a través de la cual se inyecta el complejo DNA-Relaxosoma a la célula receptora ha sido matizada por diversas evidencias experimentales. Por un lado, se han aislado mutantes deficientes en la elongación del pili en que la transferencia conjugativa no se ve afectada, insinuando que basta con la estructura basal de éste para llevarla a cabo (Eisenbrandt *et al.,* 2000). Por otro lado, experimentos de seguimiento *in vivo* de la movilización de DNA han demostrado que ésta ocurre incluso entre células distantes entre sí hasta por 12 μm, revelando que el pili es capaz de transportar DNA para su movilización (Babic *et al.,* 2008)

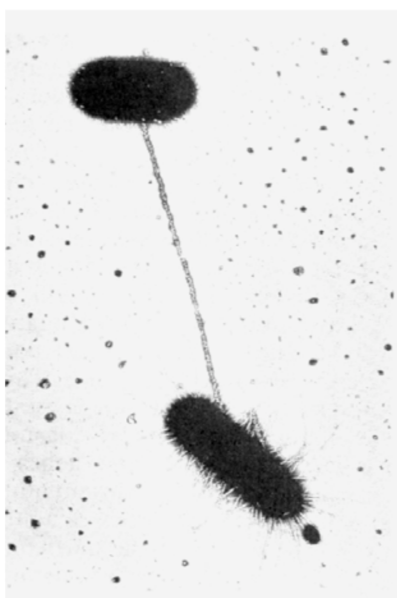


Figura 2.- Par conjugativo (www.biology200.gsu.edu)

Regulación de la Transferencia Conjugativa

La expresión de los genes de Dtr y Mpf se encuentra coordinada y varía de un plásmido a otro. Dada la carga metabólica que significa la expresión de todos los genes de transferencia, la transcripción de los mismos generalmente se encuentra firmemente regulada (Bingle y Thomas, 2001).

Uno de los mecanismos de regulación más utilizados es el de inducción dependiente de densidad celular (quorum sensing) ya que de esta forma se asegura la presencia de células receptoras. Por ejemplo, la transferencia de los plásmidos tipo *incF* (como el factor F en *Escherichia coli*) se encuentra regulada por el RNA del gen *finP*. Este funciona como RNA antisentido y bloquea la traducción del activador transcripcional, *traJ*, de manera constitutiva. La activación de un sistema de dos componentes tipo quorum sensing mejora la expresión de *traJ* a través de la expresión de *traM,* de manera que escapa de la regulación negativa de *finP,* permitiendo así la expresión el sistema de conjugación (Zechner *et al.,* 1998).

Otro sistema que es regulado por un sistema de quorum sensing es el sistema *tra* del plásmido Ti *de A. tumefaciens*. La activación de los genes *tra* de este plásmido depende de una acil homoserín lactona (N-(3-oxo-octanoil)-l-homoserina lactona) llamada inductor de *Agrobacterium* (IA) y de TraR, un activador transcripcional de la familia LuxR. Cuando este último se une con el IA se convierte, de su conformación monomérica inactiva, a su conformación dimérica activa. Esta forma activa reconoce y se une a la caja *tra*, una secuencia invertida repetida de 18 pb localizada en la región -35 del promotor de los genes *tra*, activando su transcripción (Piper *et al.*, 1999).

Adicionalmente, la transferencia conjugativa del plásmido Ti posee un nivel superior de regulación que responde a un indicador de infección efectiva. La expresión de *traR* se encuentra reprimida de forma constitutiva por AccR, un represor transcripcional responsivo a opinas. Las opinas son compuestos de bajo peso molecular que son secretados por los tumores inducidos por la infección de *A. tumefaciens*. Estas son sintetizadas por proteínas codificadas en el T-DNA que la bacteria inyecta en la planta. En presencia de opinas, AccR cesa su represión sobre *traR* y se sintetiza suficiente proteína para responder en caso de que los niveles de IA se

eleven. Diferentes plásmidos Ti de diferentes cepas de *Agrobacterium* pueden responder a otros compuestos (octopinas) pero el patrón de regulación es el mismo (Piper *et al.,* 1999).

El sistema *vir*, encargado de la movilización del T-DNA de *A. tumefaciens* es inducido, bajo condiciones ácidas, por moléculas señal de plantas (como compuestos fenólicos y monosacáridos). Estos interactúan con un sistema de dos componentes formados por *virA* y *virG*. VirA es la proteína sensora, una cinasa asociada a membrana, que al percibir las moléculas señal se auto-fosforila para después fosforilar al producto de *virG*. Esto favorece su dimerización, lo que le permite unirse a cajas *vir* en la región promotora de los genes *vir*, activando su expresión (Zechner *et al.,* 1998).

Otro caso interesante de regulación es el de los plásmidos inducidos por feromonas en *Enterococcus faecalis.* La transferencia conjugativa de estos plásmidos es inducida por péptidos de 8 aminoácidos, generados a partir del procesamiento de péptidos señal de lipoproteínas, cuyos genes se encuentran codificados en el cromosoma. Para que estos péptidos-feromona sean activos, deben ser secretados de la célula y luego introducidos a esta nuevamente. En el plásmido inducible se encuentra codificada otra proteína que secuestra los péptidos-feromona, impidiendo que sean secretados y puedan inducir el sistema. Cuando en la población se encuentran células carentes de plásmidos, éstas exportan sus péptidos sin restricciones y cuando éstos penetran en las células con plásmido desajustan el equilibrio proteína secuestradora/feromona, induciendo los genes de conjugación.

En muchos de los ejemplos mencionados anteriormente la regulación funciona de forma tan estricta, que la frecuencia conjugativa detectable experimentalmente es bastante baja (Zechner *et al.,* 1998). El único grupo de plásmidos con alta frecuencia de conjugación es el de los plásmidos de tipo IncP, que tienen una frecuencia detectable cercana a 1.0. Contrariamente a lo que se pudiera pensar, esto no significa que los plásmidos de este grupo carezcan de regulación de la conjugación. La conjugación se encuentra modulada a través de los reguladores globales *korA*, *korB* y *trbA* que sincronizan los genes conjugativos con otros procesos genéticos que ocurren a una tasa continua, como la división celular y la replicación (Adamczyk y Jagura-Burdzy, 2003).

Adicionalmente a los mecanismos descritos anteriormente, los sistemas de procesamiento de DNA cuentan con un nivel adicional de autorregulación. Debido a que el origen de transferencia se localiza comúnmente cerca de la región promotora de los genes de Dtr, cuando el relaxosoma se une al *oriT* bloquea los promotores de estos genes. De este modo se garantiza que no se sinteticen más proteínas de las que son necesarias para procesar el DNA plasmídico (Zechner *et al.,* 1998).

Plásmidos Transferibles en Rhizobiaceas

Se considera que los plásmidos de las bacterias del género *Rhizobium* están relacionados evolutivamente con los de *Agrobacterium* (Romero y Brom, 2004). Por ejemplo, existe una alta identidad entre los genes de sistemas tipo *vir* y *tra* del plásmido Ti y el plásmido simbiótico de *Rhizobium sp.* NGR234 (Moriguchi *et al.,* 2001). De manera experimental, se ha comprobado la movilización de los plásmidos simbióticos de *Rhizobium sp.* NGR234 (He *et al*,, 2003), *Rhizobium leguminosarum bv. viciae* (Danino et. al., 2003) y de otros plásmidos no simbióticos en *S. meliloti* y *R. leguminosarum bv. trifolii* (Mercado-Blanco y Toro, 1996). Adicionalmente, estudios de tipo filogenético han detectado eventos de movilización de plásmidos crípticos y del plásmido simbiótico en los géneros *Rhizobium, Mesorhizobium* y *Sinorhizobium* (Wernergreen y Riley, 1999), entre diferentes aislados de *R. etli*, así como en *R. galegae* (Valdés y Piñero, 1992). La movilización de los genes simbióticos no se limita a aquellos en que estos se encuentran codificados en plásmidos, también se ha detectado movilización de las islas simbióticas de *Bradyrhizobium japonicum* y *Mesorhizobium loti* (Kaneko *et al.,* 2000 y Sullivan *et al.,* 2002).

Conjugación en *Rhizobium etli*

Rhizobium etli CFN42 es una bacteria Gram-negativa que establece una relación simbiótica con el frijol (*Phaseolus vulgaris*). Su genoma está compuesto por un cromosoma y seis plásmidos (p42a, p42b, p42c, p42d, p42e y p42f). Estos son requeridos para llevar a cabo varias actividades

biológicas como la fijación de nitrógeno (Brom *et al.,* 2000). Solo dos de estos plásmidos poseen sistemas conjugativos:

El plásmido p42a posee un sistema *tra*/*trb* y es auto-movilizable a una frecuencia de $1x10^{-2}$. Este sistema está regulado por quorum sensing a partir de una acyl-homoserin-lactona sintetizada por el gen *traI,* en conjunto con el activador transcripcional codificado por *traR* (Tun-Garrido *et al.,* 2003).

El plásmido p42d posee un sistema *virB/tra*. En el caso de los genes de Dtr (Fig. 3), están presentes dos proteínas accesorias (*traC* y *traD*) y una relaxasa (*traA*). También está presente una proteína de interfase tipo *traG* (*traG*).

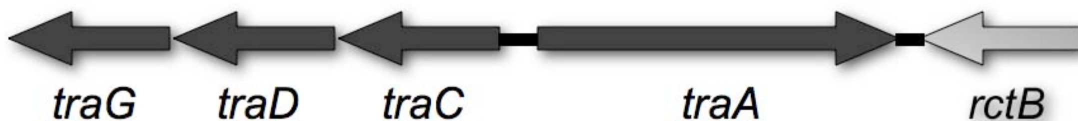


Figura 3.- Genes de Procesamiento de DNA del p42d

En el caso de los genes del complejo Mpf (Fig. 4) está presente todo el operón *virB* (*virB1-virB11*). Las secuencias, tanto de los genes *tra* como de los *vir,* poseen una mayor identidad con los de los sistemas presentes en *S. meliloti* (pSme1021a) y *A. tumefaciens* C58 (pAtC58) que con los que posee el p42a. Esto no es extraño ya que se considera que el plásmido simbiótico y el p42a son de reciente adquisición en esta especie, por lo que podrían tener diferente origen (González *et al.,* 2006).

El operón *virB* parece incorporar otro gen (*yhd0053*) que solo esta reportado en estas dos especies. Adicionalmente, no se encontró ningún gen homólogo a los sistemas tradicionales de regulación de la transferencia conjugativa en otras rhizobaceas.

Figura 4.- Genes de formación de par conjugativo del p42d

Sin embargo la movilización del p42d solo es detectable en presencia del plásmido p42a. Esto se debe a que estos plásmidos son capaces de formar un cointegrado que es movilizado por el sistema *tra*/*trb*. La cointegración se lleva a cabo de manera especifica, a través de una integrasa, pero también puede hacerse de manera inespecífica, a través de *recA*. Una vez que el cointegrado es transferido a la célula receptora, los dos plásmidos se separan por medio de recombinación. Dado que la transferencia del p42d depende de dos eventos (cointegración con p42a más transferencia del cointegrado) la frecuencia detectada de transferencia de p42d es menor (frecuencia de 2 x 10^{-6}) que la de p42a (frecuencia de 1x10^{-2}). En ausencia del p42a o de alguno de los dos mecanismos de cointegración no se detectaba la movilización del p42d (Brom *et al.,* 2004). Estos resultados parecían indicar que los genes conjugativos del plásmido simbiótico no eran funcionales o que se encontraban reprimidos por un sistema regulatorio hipotético.

ANTECEDENTES

Buscando localizar genes reguladores de la transferencia del pSym, Pérez-Mendoza y colaboradores realizaron una mutagénesis al azar, con el transposón *Tn5,* a una cepa de *R. etli* curada del plásmido p42a. Las mutantes obtenidas fueron usadas como donadoras en cruzas con *A. tumefaciens* GMI9023 y las transconjugantes fueron seleccionadas para la transferencia del marcador de resistencia del transposón. Ésta se detectó a una frecuencia de 1 x 10^{-4}, y en todos los casos estaba ligada a la adquisición del p24d por las células receptoras. Dado que las mutantes por inserción de *Tn5* adquieren la capacidad de transferir el pSym en ausencia del p42a, es de suponerse que la inserción del transposón afectó la actividad de los genes encargados de la regulación de la transferencia. Al mapear el sitio de inserción de *Tn5,* se encontró que en todas las mutantes ésta había ocurrido en uno de dos genes: *rctA* o *rctB* (Pérez-Mendoza *et al.*, 2004).

rctA, es un gen localizado adyacente al operón *virB* (Fig. 3), se encuentra conservado y en el mismo contexto genético en los plásmidos pAtC58 de *A. tumefaciens* y pSme1021a de *S. meliloti*. Su producto tiene 123

aminoácidos, un peso molecular de 13782 daltones y un punto isoeléctrico de 7.48. Un análisis de estructura terciaria predice que su producto posee un dominio de unión a DNA de tipo hélice alada, una variante del dominio hélice-vuelta-hélice. El gen *rctB* está localizado cerca de los genes *tra* (Fig. 4). Su producto tiene 156 aminoácidos, un peso molecular de 16986 daltones y un punto isoeléctrico de 5.15. También se encuentra conservado en los plásmidos pAtC58 de *A. tumefaciens* y pSme1021a de *S. meliloti*, aunque con mayor grado de divergencia que en el caso de *rctA*. No se pudo asignar un dominio o una función conocida al producto de este gene. Ninguno de estos dos genes tiene similitud con los de sistemas clásicos de regulación de la conjugación, como los sistemas de dos componentes del quorum sensing. Por lo tanto, la caracterización de la regulación transcripcional de este sistema aportaría nuevos elementos al conocimiento sobre la transferencia conjugativa en *rhizobaceas* y abriría la posibilidad de entender las señales y elementos necesarios para la transferencia del fenotipo simbiótico en la naturaleza.

OBJETIVO

- Caracterizar la regulación transcripcional del operón *virB* del plásmido simbiótico de *R. etli*.

Objetivos Particulares

- Establecer si el gen *yhd0053* forma parte del operón *virB*
- Determinar el papel de *rctA* y *rctB* en la regulación del operón *virB*.
- Caracterizar la capacidad de unión a DNA de RctA y establecer su relación con la regulación transcripcional de la transferencia conjugativa.

RESULTADOS

- Daniel Pérez-Mendoza, **Edgardo Sepúlveda**, Victoria Pando, Socorro Muñoz, Joaquina Nogales, José Olivares, Maria J. Soto, José A. Herrera-Cervera, David Romero, Susana Brom, and Juan Sanjuán. (2005) Identification of the *rctA* gene, which is required for repression of conjugative transfer of rhizobial symbiotic megaplasmids. J. Bacteriol. 187(21):7341-7350.

    - La aportación de nuestro grupo consistió en el diseño y construcción de las fusiones transcripcionales de los promotores de *rctA* y del operón *virB*, así como de los ensayos de expresión. Nuestros resultados permitieron establecer el papel que *rctA* y *rctB* juegan en la regulación de la transferencia conjugativa.

- **Edgardo Sepúlveda**, Daniel Pérez-Mendoza, Miguel A. Ramírez-Romero, María J. Soto, Isabel M. López-Lara, Otto Geiger, Juan Sanjuán, Susana Brom, and David Romero. (2009) Transcriptional interference and repression modulates conjugative ability of the symbiotic plasmid of *Rhizobium etli*. J. Bacteriol. 190(12):4189-4197.

    - En este trabajo caracterizamos la actividad de unión a DNA de RctA y la manera en que esta regula la transcripción del operón *virB*. Adicionalmente logramos develar un nivel extra de regulación para el sistema, consistente el la interferencia transcripcional que el promotor de *virB* ejerce sobre el de *rctA*.

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Identification of the *rctA* Gene, Which Is Required for Repression of Conjugative Transfer of Rhizobial Symbiotic Megaplasmids†

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An analysis of the conjugative transfer of pRetCFN42d, the symbiotic plasmid (pSym) of *Rhizobium etli*, has revealed a novel gene, *rctA*, as an essential element of a regulatory system for silencing the conjugative transfer of *R. etli* pSym by repressing the transcription of conjugal transfer genes in standard laboratory media. The *rctA* gene product lacks sequence conservation with other proteins of known function but may belong to the winged-helix DNA-binding subfamily of transcriptional regulators. Similar to that of many transcriptional repressors, *rctA* transcription seems to be positively autoregulated. *rctA* expression is greatly reduced upon overexpression of another gene, *rctB*, previously identified as a putative activator of *R. etli* pSym conjugal transfer. Thus, *rctB* seems to counteract the repressive action of *rctA*. *rctA* homologs are present in at least three other bacterial genomes within the order *Rhizobiales*, where they are invariably located adjacent to and divergently transcribed from putative *virB*-like operons. We show that similar to that of *R. etli* pSym, conjugative transfer of the 1.35-Mb symbiotic megaplasmid A of *Sinorhizobium meliloti* is also subjected to the inhibitory action of *rctA*. Our data provide strong evidence that the *R. etli* and *S. meliloti* pSym plasmids are indeed self-conjugative plasmids and that this property would only be expressed under optimal, as yet unknown conditions that entail inactivation of the *rctA* function. The *rctA* gene seems to represent novel but probably widespread regulatory systems controlling the transfer of conjugative elements within the order *Rhizobiales*.

Rhizobia are gram-negative soil bacteria that are able to establish nitrogen-fixing symbiotic associations with leguminous plants. Besides the chromosome, their genomes are usually constituted of large plasmids which carry genetic material relevant for diverse functions. In some species, the plasmid contribution to the total genome size is certainly astonishing (from 25 to 50% of the genome size [20, 42]). Most of the genes for nodulation and nitrogen fixation are usually located in one of these large replicons, known as symbiotic plasmids (pSyms), or clustered in "symbiosis islands" within the chromosome. In other cases, the participation of another plasmid in symbiosis is considered important enough to justify the denomination of pSym. For example, *Sinorhizobium meliloti* carries two megaplasmids: pSymA (1.35 Mb), the "true" pSym, contains most of the genes needed for nodulation and nitrogen fixation, whereas pSymB (1.68 Mb) harbors exopolysaccharide biosynthetic genes (19), which are also required for the establishment of symbiosis.

Advances in the knowledge of the transfer systems of these pSyms have been hindered by some particular characteristics of these replicons, such as their large size and the difficulty of detecting their transfer under laboratory conditions. However, there is abundant evidence of symbiotic plasmid transfer among these bacteria in soil (11, 26, 50, 56, 58). In addition, genome sequencing has revealed that many rhizobial pSyms carry genes potentially involved in conjugal transfer (19, 22, 46). For example, putative *D*NA *t*ransfer and *r*eplication genes (*dtr*) involved in the processing of plasmid DNA during conjugative transfer have been identified in several rhizobial pSyms, including pSymA of *S. meliloti* (2), pRetCFN42d (pSym of *Rhizobium etli* [22]), and pNGR234a (pSym of the broad-host-range strain *Rhizobium* sp. strain NGR234 [46]). These Dtr functions are commonly encoded by two divergently transcribed operons, *traA* and *traCDG*, with a hypothetical origin of conjugative transfer (*oriT*) in the intergenic regions (reviewed in reference 52).

Other genes likely involved in the conjugal transfer of pSym plasmids have also been identified, such as complete *trb*-like systems (in pNGR234a [46] and pRL1JI, the pSym of *Rhizobium leguminosarum* bv. viciae [59]) or *virB*-like (2, 22) type IV secretion systems (T4SS), which potentially code for *m*ating *p*air *f*ormation (Mpf) systems required for the transfer of DNA across the membranes of donor and recipient cells during conjugation (52).

Despite the evidence indicating the potential of these plasmids for conjugation, their transfer under laboratory conditions is usually undetectable or occurs at negligible frequencies. Uncovering the regulatory networks that likely control the conjugative transfer of most of these plasmids is one of the

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keys to identifying the environmental conditions promoting the transfer of these symbiotic megaplasmids and to better understanding the role of pSym lateral spread in rhizobial ecology.

In *Agrobacterium*, a genus of the *Rhizobiaceae* family, conjugal transfer of the tumor-inducing plasmid (pTi) is a quorum sensing-dependent highly regulated process (5, 31). Opines produced by the plant during infection control transcription from at least five promoters in gene operons involved in plasmid transfer (38, 47). Some rhizobial symbiotic and nonsymbiotic plasmids with quorum-sensing conjugal transfer regulation systems have been reported as well (15, 25, 27, 28, 40, 57). However, other rhizobial pSyms, such as pRetCFN42d of *R. etli* CFN42 and pSymA of *S. meliloti* 1021, seem to have no quorum sensing-like genes carried in their genomes (2, 22). This suggests that these plasmids either have different regulatory systems controlling their transfer or, as suggested previously (22), may have lost the capacity for efficient lateral spread.

The presence of *S. meliloti* pSymA has been described as an ancient event of lateral transfer mediated by import of pSymA from an unknown bacterium, as indicated by its distinctive G+C content and codon usage (19), but this plasmid has never been described as self-transmissible under laboratory conditions.

In *R. etli*, the ability of the pSym pRetCFN42d to cointegrate with the accompanying conjugative plasmid pRetCFN42a provides an alternative means for lateral spread (10, 12, 57). However, Pérez-Mendoza and coworkers have recently identified a gene (yp028) involved in the promotion of *R. etli* pSym transfer at relatively high frequencies in standard media in the absence of pRetCFN42a (45). The identification of this gene opened the possibility that conjugal transfer functions encoded by the *R. etli* pSym might be silent under laboratory conditions and that transfer could be activated with the participation of yp028 under as yet unknown conditions.

Here we report the identification of the *rctA* gene present in the symbiotic plasmids of *R. etli* and *S. meliloti*. *rctA* prevents the transfer of these two megaplasmids in standard media and is required for the repression of conjugal transfer genes under these conditions. The existence of this novel regulatory system to actively limit pSym transfer under certain conditions further supports our interpretation that conjugative transfer of these megaplasmids may be a highly regulated process responding to as yet unknown signals.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacteria and plasmids used for this work are listed in Table 1. *R. etli* and *S. meliloti* strains were grown at 30°C on TY (tryptone-yeast extract-$CaCl_2$ [6]). *Escherichia coli* and *Agrobacterium tumefaciens* were grown on Luria-Bertani (LB) medium (54). When required, antibiotics were added at the following concentrations: nalidixic acid (Nx), 20 μg/ml; spectinomycin (Spc), 100 μg/ml for *R. etli* and 200 μg/ml for *S. meliloti*; kanamycin (Km), 50 μg/ml for *R. etli* and *A. tumefaciens* and 200 μg/ml for *S. meliloti*; gentamicin (Gm), 10 μg/ml; rifampin, 50 μg/ml; streptomycin (Sm), 100 μg/ml for *R. etli* and *A. tumefaciens*, 200 μg/ml for *S. meliloti*, and 25 μg/ml for *E. coli*; and tetracycline (Tc), 2 μg/ml for *Rhizobium* and 10 μg/ml for *E. coli*.

Bacterial matings. Donor strains, grown to an approximate optical density at 600 nm (OD_{600}) of 0.2, and recipient strains, grown to late exponential phase, were washed and mixed at a 1:1 ratio of donor to recipient. Mating mixtures were resuspended in 50 μl of TY medium and loaded onto a sterile nitrocellulose filter with a 0.45-μm pore size. Filter mating mixtures were deposited on TY-agar plates and incubated overnight at 30°C. Cells were resuspended by vigorous vortexing and diluted in liquid medium. To calculate transfer frequencies, donor, recipient, and transconjugant CFU were counted after mating disruption and plating of serial dilutions. Transconjugants were selected on plates supplemented with appropriate antibiotics. The transfer frequency was expressed as the number of transconjugants per output recipient. Donor and recipient levels of spontaneous resistance to selective antibiotics were also determined.

Cell transformations. Bacterial transformation was carried out by electroporation using an electrocell manipulator apparatus (BTX 600). Electrocompetent cells were prepared according to the instructions of the manufacturer and then stored at −80°C. For electroporation, cells were thawed on ice, mixed with plasmid DNA (0.3 to 0.5 μg/ml of cell suspension), and then transferred to a 0.2-cm-electrode-gap chilled cuvette. A pulse with a 2.5-kV/cm field strength, a 6.8-ms length, and a 129-Ω set resistance was applied, and cells were immediately suspended in 1 ml of TY or LB medium and then incubated at 30°C for 15 h (*R. etli*) or at 37°C for 1 h (*E. coli*). Appropriate dilutions were plated on selective media.

Plasmid profiles. Plasmid profiles were visualized by the Eckhardt procedure (16), as modified by Hynes and McGregor (32).

DNA hybridizations. Total genomic DNAs were isolated by standard procedures (41), digested with the endonucleases EcoRI and BamHI, electrophoresed in 0.8% agarose gels, and then transferred to positively charged nylon membranes by the method of Southern (54). DNA hybridization probes were labeled with digoxigenin according to the manufacturer's instructions (Roche, Barcelona, Spain). Hybridization and membrane washes were carried out under high-stringency conditions. Membranes were prepared for chemiluminescence detection (Roche) and exposed to Kodak X-Omat film (Sigma).

PCR, cloning, and sequencing. Standard DNA techniques were used as described previously by Sambrook et al. (54). The Tn*5* insertions of the different mutants were subcloned into pUC18 as EcoRI fragments. Transposon arms were subcloned, and insertion sites were mapped by DNA sequencing using an IS*50*-specific primer. For each insertion, sequence stretches of between 600 nucleotides (nt) and >1,000 nt were obtained. Sequencing was performed with an ABI 373 automated sequencer. DNA sequence editing, translation, and analysis were performed with the Vector NTI 5.5 software package (Oxford Molecular) and the program BLAST from the network service at NCBI (1). The RctA winged-helix DNA-binding domain was identified using the program Superfamily 1.65 (23; http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/). Three-dimensional prediction structures of proteins were prepared using the 3D-JIGSAW program (version 2.0) (3, 4, 14; http://www.bmm.icnet.uk/servers/3djigsaw/).

All oligonucleotides used for constructions are listed in Table S1 in the supplemental material. Different gene fusions to the β-glucuronidase reporter gene *gusA* were constructed by cloning the pRetCFN42d *traA-traC* and yp037-*rctA* DNA regions into the vector p53gus. Primers UptraAE and LwtraCE were used to amplify a 1,394-bp DNA fragment containing the *traC* promoter, and primers UptraAXb and LwtraCK were used to amplify a 1,363-bp DNA fragment containing the *traA* promoter. These two PCR fragments were cloned into a dephosphorylated blunt-ended pMOS*Blue* vector. Among the two possible orientations, the correct one was identified by PCR using UptraA− (E or Xb) and the vector-specific primer T7 and then cloned as a SpeI/KpnI (in the p53Re*traC*::*gus* construct) or XbaI/KpnI (in the p53Re*traA*::*gus* construct) fragment into p53gus, which was previously digested with the corresponding restriction enzymes. p53Re*yp037*::*gus* and p53Re*yp038*::*gus* were constructed by cloning the PCR fragments containing the yp037 promoter (amplified with yp037up and yp037lw) and the yp038 promoter (amplified with yp038up and yp038lw) into p53gus, which was previously digested with XhoI/XbaI (restriction enzyme sites are underlined in the primer sequences).

S. meliloti gene fusion plasmids were constructed by cloning a *traA1-traC* fragment of 480 bp, previously amplified with primers SmtraACup and SmtraA-Clw, into the pGEM-T Easy cloning vector. Using the XbaI, EcoRI, and SalI restriction enzyme sites at the 5′ ends of the primers, different fragment orientations were obtained with respect to the *gusA* reading frame in p53gus. All p53gus derivatives were introduced into rhizobial strains by conjugation using the S17-1 mobilizing strain.

For complementation experiments, different plasmids derived from pTE3 were constructed. The different *rctA* alleles were amplified using specific primers with restriction sites at their 5′ ends to facilitate subsequent cloning in front of the *trp* promoter in pTE3. pTEYp038 contains the *rctA* gene of pRetCFN42d as a 750-bp fragment previously amplified with primers RerctA-F and RetrctA-R. pTEAtu5160 contains the *rctA* gene of pAtC58 as a 450-bp fragment that was amplified with the primers AtrctA-F and AtrctA-R. pTESMa1323 contains the *rctA* gene of pSymA as a 430-bp fragment that was obtained after amplification with primers SmrctA-F and SmrctA-R.

Construction of an *S. meliloti* pSymA SMa1323 mutant. The oligonucleotides used to construct the pSymA SMa1323 mutant are listed in Table S1 in the supplemental material. Two pairs of primers, SmrctA1-SmrctA2 and SmrctA3-SmrctA4,

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant features	Reference or source
Strains		
S. meliloti		
1021	Wild-type strain, Sm^r	41
1021 RctA$^-$	1021 derivative (SMa1323::Sm/Spc)	This work
10OTSS	1021 derivative (Δ*otsA*::Sm/Spc)	A. Dominguez (EEZ)
R. etli		
CE3	Sm^r derivative of wild-type strain CFN42	49
CFNX182	CE3 derivative cured of p42a	9
CFNX218Spc	CE3 derivative (p42a$^-$ p42b$^-$ p42c$^-$ p42d$^-$ p42eΔ p42f) Spc^r	This work
CFNX218Spc Tn5.1	CFNX218Spc derivative with pRetCFN42d::Tn5.1	This work
CFNX218Spc Tn5.2	CFNX218Spc derivative with pRetCFN42d::Tn5.2	This work
CFNX218Spc Tn5.6	CFNX218Spc derivative with pRetCFN42d::Tn5.6	This work
CFNX218Spc Tn5.8	CFNX218Spc derivative with pRetCFN42d::Tn5.8	This work
CFNX218Spc Tn5.13	CFNX218Spc derivative with pRetCFN42d::Tn5.13	This work
A. tumefaciens		
C58	Wild-type, nopaline-resistant strain	63
GMI9023	Plasmidless C58 derivative	53
At Tn5.1	GMI9023 derivative with pRetCFN42d::Tn5.1	This work
At Tn5.2	GMI9023 derivative with pRetCFN42d::Tn5.2	This work
At Tn5.6	GMI9023 derivative with pRetCFN42d::Tn5.6	This work
At Tn5.8	GMI9023 derivative with pRetCFN42d::Tn5.8	This work
At Tn5.13	GMI9023 derivative with pRetCFN42d::Tn5.13	This work
E. coli		
DH5α	*supE44 Dlac U169 f80 lacZDM 5hsdR171 recA1 endA1 gyrA96 thi-1 relA1*	24
S17-1	*thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn7	55
Plasmids		
pTE3	IncP cloning vector carrying *Salmonella enterica* serovar Typhimurium *trp* promoter, Tc^r	17
pMOS *Blue*	PCR cloning vector, Ap^r	Amersham
pGem-T Easy	PCR cloning vector, Ap^r	Promega
pSUP202	Mobilizable Tc^r Cm^r Ap^r suicide vector	55
pHP45Ω	pBR322 derivative with the Sm/Spc cassette; Sm^r Spc^r Ap^r	48
pUC18	2,690-bp cloning vector, Ap^r	61
p53Gus	pBBR1MCS5 derivative with a *gus* gene of pWM5 (pBBR1MCS5::*uidA*)	L. Girard (CCG, México)
p53Re*traA*::*gus*	pRetCFN42d *traA*::*gus* fusion in p53Gus	This work
p53Re*traC*::*gus*	pRetCFN42d *traC*::*gus* fusion in p53Gus	This work
p53*yp037*::*gus*	pRetCFN42d *yp037*::*gus* fusion in p53Gus	This work
p53*yp038*::*gus*	pRetCFN42d yp038::*gus* fusion in p53Gus	This work
p53SmtraA1::*gus*	*S. meliloti* 1021 *traA1*::*gus* fusion in p53Gus	This work
p53SmtraC::*gus*	*S. meliloti* 1021 *traC*::*gus* fusion in p53Gus	This work
pTEYp028	pTE3 with yp028 cloned in front of *trp* promoter, yp028 (Con)	45
pTEYp038	pTE3 with yp038 cloned in front of *trp* promoter, yp038 (Con)	This work
pTEAtu5160	pTE3 with Atu5160 cloned in front of *trp* promoter	This work
pTESMa1323	pTE3 with SMa1323 cloned in front of *trp* promoter	This work

were used to amplify two DNA fragments which flanked the *S. meliloti* 1021 SMa1323 gene. Each PCR product was cloned into pUC18, the R fragment was cloned as a SmaI/XbaI fragment, generating pUC18-R, and the L fragment was cloned as a BamHI/HindIII fragment, generating pUC18-L. A triple ligation reaction included an Sm/Spc resistance gene cassette (a BamHI fragment purified from the pHP45Ω vector), the R fragment (as a BamHI/HindIII fragment purified from pUC18-R), and pUC18-L (previously linearized with BamHI/HindIII). The resulting construct (Ap, Sm, and Spc resistant; pUC18LCR) was restricted with SmaI/EcoRV, and the corresponding LCR fragment (containing the fragment L plus the resistance Cassette plus R) was cloned into suicide plasmid pSUP202 and introduced by conjugation into strain 1021 of *S. meliloti*. Allele replacement events were selected as described previously (55), and *rctA* mutants were verified after hybridization with a labeled plasmid, pUC18LCR, as a probe.

Gene expression assays. Rhizobial strains containing reporter gene fusion plasmids were grown in TY selective medium to an approximate OD_{600} of 0.8. Cultures were diluted (1/200 to 1/500) in TY medium containing 20 μg/ml Gm to an approximate OD_{600} of 0.4 to 0.6. Cells in 1.5 ml of medium were pelleted, washed three times with sterile distilled water to remove any traces of TY, and finally resuspended in 1.5 ml of assay buffer (dithiothreitol, 5 mM; EDTA, 1 mM; Na_2HPO_4-NaH_2PO_4, 50 mM). Three hundred microliters of this sample was stored at −20°C for the Bradford Bio-Rad protein assay. Another 200 μl was used to determine the β-glucuronidase activity by mixing with 740 μl of assay buffer, 50 μl of 0.1% sodium dodecyl sulfate, and 100 μl of chloroform and vortexing twice for 15 seconds each time. Samples were incubated at 37°C for 10 min, and then 10 μl of 100 mM NPG (4-nitrophenyl-β-D-glucuronide), preheated at 37°C, was added. Samples were incubated at 37°C until they turned yellow. The reactions were ended by adding 200 μl of 1 M Na_2CO_3. Samples were centrifuged for 5 min (12,000 rpm), and the absorbance of the upper phase of each sample was measured at 405 nm with a spectrophotometer. Values of β-glucuronidase activity were expressed as specific β-glucuronidase activities (nmol/min/mg of protein). Mean values and standard deviations were calculated from at least three independent experiments.

RESULTS

Isolation and genetic characterization of conjugative transfer regulatory elements in the *R. etli* symbiotic plasmid. The

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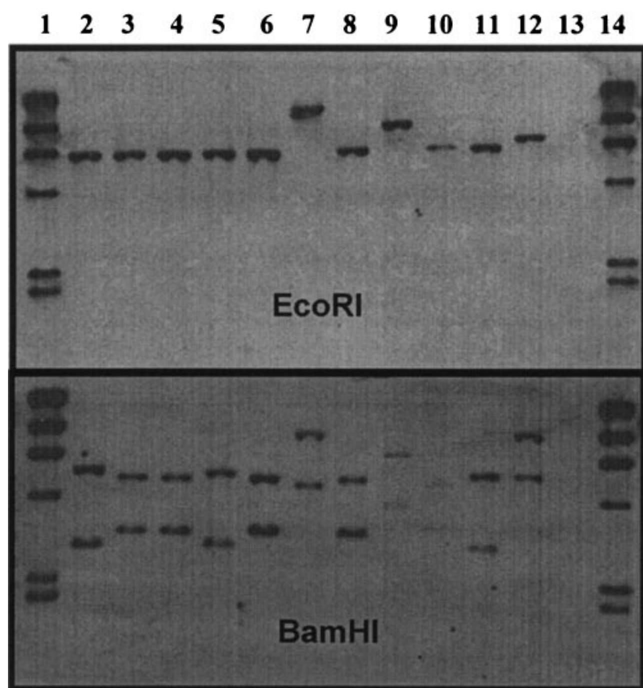

FIG. 1. Southern blots of EcoRI- and BamHI-digested genomic DNAs of 11 *A. tumefaciens* GMI9023 Km^r transconjugants, with digoxigenin-labeled Tn*5* as a probe. Lanes: 1 and 14, digoxigenin-labeled DNA molecular weight marker; 2 to 12, transconjugants harboring a Tn*5* insertion; 13, *A. tumefaciens* GMI9023.

TABLE 2. Transfer frequencies of different pRetCFN42d Tn5 mutant derivatives from a plasmidless *A. tumefaciens* strain to *R. etli* CFNX218Spc

Mutant plasmid	Frequency of transfer[a] to *A. tumefaciens* GMI9023
pRetCFN42d::Tn*5*Mob	ND
pRetCFN42d::Tn*5*.1	1.48×10^{-3}
pRetCFN42d::Tn*5*.2	1.94×10^{-3}
pRetCFN42d::Tn*5*.6	3.14×10^{-4}
pRetCFN42d::Tn*5*.8	3.81×10^{-7}
pRetCFN42d::Tn*5*.13	2.13×10^{-7}

[a] Expressed as the number of transconjugants per input receptor cell. Frequencies are the averages of at least three independent experiments. pRetCFN42d::Tn*5*Mob is a pRetCFN42d Km^r derivative used as a control. ND, no transconjugants detected.

symbiotic plasmid of *R. etli* CFN42, pRetCFN42d, has never been shown to be self-transmissible. However, data from a previous work indicated that conjugative transfer of this pSym may be silent under laboratory conditions, suggesting the possibility of an active repression system in this plasmid (45). In order to identify putative genes participating in the repression of *R. etli* pSym conjugal transfer, strain CFNX182 of *R. etli* (pRetCFN42a$^-$) was subjected to random Tn*5* mutagenesis using the suicide vector pSUP2021 (55). About 2.2×10^6 transposants were obtained from 10 independent matings, where each CFNX182 Km^r transconjugant should harbor a single Tn*5* insertion in its genome. The pool of CFNX182 transposants was used as a donor en masse in conjugation with the plasmidless *A. tumefaciens* GMI9023 strain as the recipient. Transposon insertions causing a loss of functionality of a hypothetical repressor of conjugative plasmid functions would result in a relief of repression and therefore would allow plasmid transfer. Tn*5* insertions able to promote the transfer of any plasmid in strain CFNX182 could be easily identified among *A. tumefaciens* GMI9023 Km^r transconjugants. Some 400 GMI9023 transconjugants were obtained from such mating. Plasmid profiles of 11 randomly chosen transconjugants showed that they all carried a plasmid with a similar size to that of the *R. etli* symbiotic plasmid pRetCFN42d. Southern hybridization analysis of digested genomic DNAs revealed that each transconjugant harbored a single copy of Tn*5* in its genome (Fig. 1). Transposon insertions could be grouped into five different types, which were named Tn*5*.1, Tn*5*.2, Tn*5*.6, Tn*5*.8, and Tn*5*.13. These results suggested that different Tn*5* insertions were producing the hypothetical derepression of plasmid transfer and that most, if not all, such insertions seemed to have occurred on the pSym plasmid pRetCFN42d but not on any other of the four additional plasmids carried by *R. etli* CFNX182.

To verify that the mutant pRetCFN42d plasmids had indeed gained self-transmissibility, the five different types of *A. tumefaciens* GMI9023 derivatives were then individually used as donors for conjugation with *R. etli* CFNX218Spc. All the Tn*5* insertions promoted transfer of the Tn*5*-encoded Km resistance from *A. tumefaciens* to *R. etli*, but at three different frequencies (Table 2). Insertions Tn*5*.1 and Tn*5*.2 promoted transfer at relative high frequencies under laboratory conditions (about 10^{-3} transconjugants per recipient cell), whereas the Tn*5*.6 mutant plasmid was transferred at a ninefold lower frequency than the Tn*5*.1 or Tn*5*.2 insertion (about 10^{-4}); insertions Tn*5*.8 and Tn*5*.13 also promoted the transfer of pRetCFN42d, but at much lower frequencies (Table 2). Plasmid profiles of the various transconjugant types confirmed the transfer of the Tn*5*-tagged pRetCFN42d mutants in all cases. Individual *R. etli* transconjugants arising from each of the later matings were again used as donors in new matings with *A. tumefaciens* GMI9023 as the receptor strain. The conjugal transfer frequencies from the individual *R. etli* CFNX218Spc donors were similar to those previously obtained from the *A. tumefaciens* GMI9023 donors (data no shown). These results confirmed that Tn*5* insertions harbored by pRetCFN42d and selected with our strategy promoted conjugative transfer of this plasmid in standard laboratory media without the help of any other *R. etli* plasmid, in contrast to the wild-type plasmid, which shows no detectable self-transfer under these conditions (10, 45).

The EcoRI fragments from the mutant plasmids containing the transposon insertions promoting high-frequency transfer of pRetCFN42d, i.e., Tn*5*.1, Tn*5*.2, and Tn*5*.6, were cloned, and the Tn*5* flanking DNAs were sequenced. Sequence comparison with the pRetCFN42d genome sequence allowed the identification of the DNA regions interrupted by Tn*5*. Insertions Tn*5*.1 and Tn*5*.2 were located within the same EcoRI fragment but were 67 bp apart. In both mutants, the transposon had interrupted the coding sequence of the yp038 gene (Fig. 2), located adjacent to a hypothetical *virB*-like operon present in pRetCFN42d. In contrast, insertion Tn*5*.6 was mapped upstream of the yp028 open reading frame (Fig. 2). yp028 is located downstream of *traA*, the hypothetical plasmid relaxase

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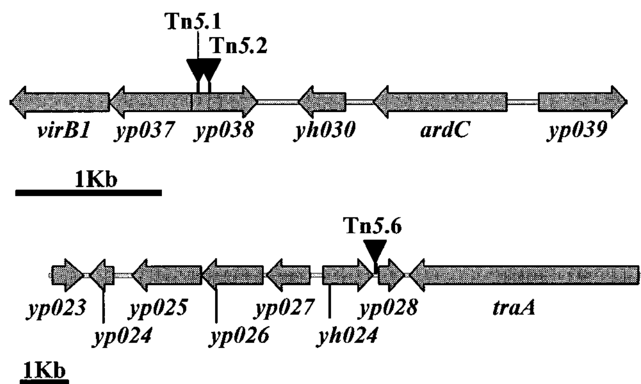

FIG. 2. Locations of different Tn*5* insertions in the pRetCFN42d genome. Transposon positions are shown as black triangles.

gene. Recently, yp028 was described as a gene which may act in the activation of conjugal transfer of the *R. etli* symbiotic plasmid (45).

yp038 represses conjugal transfer of the *R. etli* symbiotic plasmid. yp038 encodes a hypothetical protein of 149 amino acids that lacks significant similarity to any protein sequence of known function described in the databases. Nevertheless, this gene displays sequence conservation with several open reading frames of unknown function present in genomes of different bacteria within the order *Rhizobiales* (see below; Fig. 3A). Using the software program Superfamily 1.65 (23, 39), a "winged-helix" DNA-binding domain was identified in the hypothetical products of yp038 and its homologues (Fig. 3A). The winged-helix DNA-binding proteins constitute a subfamily within the large ensemble of helix-turn-helix proteins (18). The three-dimensional structure predicted by the 3D-JIGSAW (version 2.0) comparative modeling program (3, 4, 14) showed for Yp038 a similar α/β topology (Fig. 3B) to that of the winged-helix domains of several crystallized transcriptional regulators (35, 62).

The high pRetCFN42d transfer frequencies observed upon interruption of yp038 (insertions Tn*5*.1 and Tn*5*.2) suggested that this gene could act by repressing conjugal transfer of the *R. etli* symbiotic plasmid. To confirm this hypothesis, *R. etli* cells harboring Tn*5*.1 and Tn*5*.2 pSym mutants were complemented in *trans* with a yp038 gene under the control of the *trp* promoter (pTEyp038). Conjugal transfer of the mutant plasmids was then tested in matings with *A. tumefaciens* GMI9023 as the recipient. In all cases, the presence of the cloned yp038

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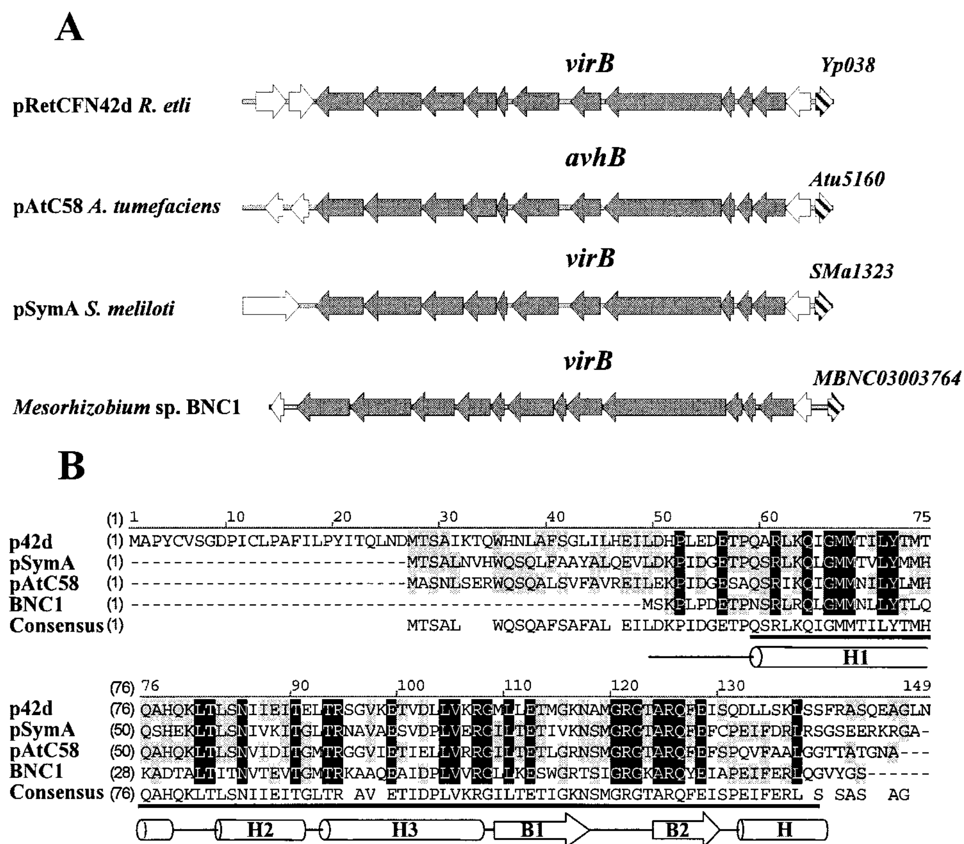

FIG. 3. (A) Genomic locations of *rctA* copies in pRetCFN42d from *R. etli*, pSymA from *S. meliloti*, pAtC58 from *A. tumefaciens*, and the chromosome of *Mesorhizobium* sp. strain BNC1. RctA open reading frames are shown as striped arrows, and *virB*-like genes are shown as gray arrows. (B) Sequence alignment of RctA homologs. Black-shaded residues are conserved in all four sequences, and gray-shaded amino acids are conserved in at least two of the four sequences. The consensus sequence is depicted below, and the putative winged-helix DNA-binding region is underlined. The α-helix and β-sheet regions of the putative winged-helix DNA-binding region of these four *rctA* copies, predicted by the 3D-JIGSAW program, are indicated with cylinders and arrows, respectively.

TABLE 3. Influence of *rctA* genotype on expression of *R. etli* pSym conjugal transfer genes

Transcriptional fusion	CFNX218 background[a]	Relevant genotype[b]	β-Glucuronidase activity[c]	Relative expression[d]
traA::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	10.41 ± 1.82	1
	p42d::Tn*5*.1	*rctA*	61.89 ± 5.90	5.94
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	5.46 ± 1.25	0.52
traC::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	9.34 ± 2.04	1
	p42d::Tn*5*.1	*rctA*	62.08 ± 7.66	6.65
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	5.30 ± 0.89	0.56
yp037::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	60.2 ± 8.1	1
	p42d::Tn*5*.1	*rctA*	282 ± 44.73	4.68
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	87.2 ± 22.4	1.44
yp038::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	332.3 ± 36.6	1
	p42d::Tn*5*.1	*rctA*	53.4 ± 4	0.16
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	354.1 ± 44.73	1.07

[a] Expression was assayed in *R. etli* strain CFNX218Spc containing pRetCFN42d::Tn*5*Mob (a pRetCFN42d Km^r derivative) or the pRetCFN42d::Tn*5*.1 mutant.
[b] *rctA*$^+$, wild-type *rctA*; *rctA*, no active *rctA; rctA*(Con), *rctA* expressed from *trp* promoter in pTE3 vector.
[c] Specific β-glucuronidase activity is expressed as nmol/min/mg of protein. Standard deviations were calculated from at least three independent experiments.
[d] Ratio of specific β-glucuronidase activity in a given background with respect to the corresponding wild-type background (p42d::Tn*5*Mob).

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gene resulted in a complete inhibition of transfer of the mutant pSyms, confirming the important role of yp038 in silencing the conjugative transfer of *R. etli* pSym. In contrast, complementation of the pRetCFN42d Tn*5*.1 mutant derivative with a yp038 gene under the control of the *trp* promoter in the *A. tumefaciens* GMI9023 background resulted in a reduction of only 2 to 3 log of the transfer frequency of the mutant plasmid but not in the complete inhibition of pSym transfer (data not shown). This result suggested that the product of the yp038 gene expressed in *trans* was not able to totally repress plasmid conjugation in the *A. tumefaciens* genetic background.

We also analyzed the effect of a yp038 mutation on the expression of pRetCFN42d conjugal transfer genes by using transcriptional reporter gene fusions. The expression of the putative Dtr (p53Re*traA*::*gus* and p53Re*traC*::*gus*) and Mpf (p53*yp037*::*gus*) genes was elevated (between 4.7- and 6.7-fold) in a yp038 mutant (pRetCFN42d::Tn*5*.1) with regard to the wild-type plasmid (Table 3). Furthermore, the expression of all of these genes returned to the wild-type levels when the yp038 mutation was complemented in *trans* with a cloned yp038 gene (pTEyp038) (Table 3). In contrast to the case for conjugal transfer genes, we observed that *rctA* expression (yp038::*gus* fusion) was greatly reduced in a RctA$^-$ background and returned to wild-type levels when a cloned *rctA* gene was provided in *trans* (Table 3). These data suggest that *rctA* expression is positively autoregulated.

These results clearly show that yp038 actively participates in keeping low expression levels of genes likely involved in the conjugal transfer of pRetCFN42d. In view of the role of yp038 in the regulation of conjugal transfer of pRetCFN42d, we have renamed this gene *rctA* (*r*egulation of *c*onjugal *t*ransfer).

Overexpression of yp028 activates the expression of pRetCFN42d conjugal transfer genes. In a previous work, we demonstrated that the overexpression of yp028 promotes the conjugal transfer of pRetCFN42d (45). Here we have found that the Tn*5*.6 insertion, located upstream of the yp028 coding sequence (Fig. 2), promotes transfer of the *R. etli* symbiotic plasmid at similar frequencies to those observed when yp028 is overexpressed (Table 2) (45).

To determine whether the overexpression of yp028 or the Tn*5*.6 transposon insertion could affect the transcription of pRetCFN42d conjugal transfer genes, we analyzed the expression of the *traA* and *traC* genes, as well as that of the yp037 gene, the hypothetical first gene of the *virB*-like operon coding for the putative Mpf of pRetCFN42d. The expression of yp028 from the *trp* promoter led to enhanced expression of hypothetical transfer genes (between 4.1- and 6.1-fold) (Table 4). Likewise, the Tn*5*.6 insertion increased the expression of the hypothetical transfer genes between four- and fivefold (data not shown). Thus, this mutation had a similar effect to that observed when the yp028 gene was overexpressed.

The location of the Tn*5* insertion upstream of the yp028 gene coding region, the observed plasmid pSym transfer frequencies, and the effect on the expression of conjugal transfer genes from pRetCFN42d allow us to propose that the Tn*5*.6 insertion may have the effect of deregulating the yp028 gene. Altogether, these results support the idea that yp028 acts as a positive element in the regulation of pRetCFN42d conjugal transfer. Therefore, we have renamed this gene *rctB*.

***rctB* has a role in reducing *rctA* expression.** As shown above, the conjugal transfer of the *R. etli* symbiotic plasmid seems to be promoted by the action of the *rctB* gene and repressed by

TABLE 4. Influence of cloned yp028 gene on expression of pRetCFN42d conjugal transfer genes

Background[a]	β-Glucuronidase activity[b]	Relative expression[c]
p53*gus* (empty vector)	7.15 ± 1.49	
p53*traA*::*gus*	10.41 ± 1.82	1
p53*traA*::*gus* + pTEYp028	42.70 ± 4.4	4.1
p53*traC*::*gus*	9.51 ± 1.88	1
p53*traC*::*gus* + pTEYp028	50.06 ± 3.23	5.26
p53*yp037*::*gus*	60.20 ± 8.1	1
p53*yp037*::*gus* + pTEYp028	369 ± 28.4	6.13
p53yp038::*gus*	332.3 ± 36.6	1
p53*yp038*::*gus* + pTEYp028	176.7 ± 28.4	0.53

[a] Expression was assayed in *R. etli* CFNX218Spc containing pRetCFN42d::Tn*5*Mob (a pRetCFN42d Km^r derivative).
[b] Specific β-glucuronidase activity is expressed as nmol/min/mg of protein. Standard deviations were calculated from at least three independent experiments.
[c] Ratio of specific β-glucuronidase activity in a given background with respect to the corresponding wild-type background (absence of pTEYp028).

the *rctA* gene. Therefore, we wondered whether the functions of these conjugal transfer regulators were somewhat related. Using a transcriptional fusion of the *rctA* gene to the *gus* reporter gene (p53*yp038*::*gus*), we analyzed the effect of *rctB* on the expression of *rctA*. As shown in Table 4, overexpression of the *rctB* gene cloned under the control of the *trp* promoter led to a significant reduction of *rctA* expression (Table 4), indicating that the *rctB* product interferes with *rctA* expression. A similar effect on *rctA* expression was observed in the pRetCFN42d::Tn*5*.6 mutant (data not shown). These results suggest that the role of *rctB* in enhancing the expression of conjugal transfer genes might be an indirect phenomenon due to a negative effect of the *rctB* product on *rctA* expression or activity.

The *rctA* gene is present in several bacteria within *Rhizobiales*. Although the predicted *rctA* gene product has no significant sequence conservation with proteins of known function, it displays sequence conservation (between 45% and 57% sequence identity; Fig. 3B) with at least three other open reading frames of unknown function present in the sequenced genomes of different bacterial species within the *Rhizobiales* order. The hypothetical products of SMa1323 located in pSymA of *S. meliloti* 1021 and of Atu5160 (from pAtC58, the cryptic plasmid of *A. tumefaciens* C58) not only show very significant sequence conservation with RctA but are also located in similar genomic contexts, divergently transcribed from *virB*-like operons carrying the hypothetical plasmid T4SS (Fig. 3A). Indeed, for *R. etli* and *S. meliloti* these genes were annotated *virB1* to *virB11* (19, 22).

A third *rctA* homolog has been identified within the unfinished genome sequence of a gram-negative bacterium that was isolated by its ability to degrade chelating compounds such as EDTA, nitrilotriacetate, and diethylenetriaminepentaacetate (34, 43, 44). This strain was initially classified as *Agrobacterium* sp. strain BNC1 (7) but was recently renamed *Mesorhizobium* sp. strain BNC1, and its genome is being sequenced by the DOE Joint Genome Institute (NZ_AAED00000000). The shotgun sequence shows that the genome of this bacterium is composed of a unique 4,922,255-bp replicon. Similar to the rhizobium and *A. tumefaciens* counterparts, the *rctA* gene homolog from this bacterium seems to be located adjacent to a hypothetical *virB*-like operon (Fig. 3A).

Recently, Chen and coworkers (13) have described the functionality of the pAtC58 T4SS, named AvhB. This system is essential for pAtC58 plasmid transfer, suggesting that AvhB products comprise an Mpf system (13). Although pAtC58 carries a *rctA* homolog (60), this plasmid displays high-frequency conjugative transfer under laboratory conditions, in contrast to the *R. etli* and *S. meliloti* pSyms (13, 53). This suggests that either the pAtC58 *rctA* copy is not functional or the genetic background may influence the activity of *rctA*.

To test whether the *rctA* homologs from *S. meliloti* pSymA and pAtC58 are functional, the putative *S. meliloti* and *A. tumefaciens rctA* homologs were placed under the control of the heterologous *trp* promoter (pTESMa1323 and pTEAtu5160) and then introduced into a pRetCFN42d RctA$^-$ mutant (pRetCFN42d::Tn*5*.1). Using *A. tumefaciens* GMI9023 as the recipient, the transfer of pRetCFN42d::Tn*5*.1 from *R. etli* CFNX218Spc became undetectable in the presence of either the pSymA or pAtC58 *rctA* homolog, showing that both of

TABLE 5. Effect of *rctA* mutation on expression of *S. meliloti* pSymA conjugal transfer genes

Transcriptional fusion	Background[a]	β-Glucuronidase activity[b]	Relative activity[c]
p53Sm*traA1*::*gus*	1021	5.03 ± 0.65	1
	1021 RctA$^-$	11.38 ± 0.59	2.26
p53Sm*traC*::*gus*	1021	70.62 ± 6.05	1
	1021 RctA$^-$	284.59 ± 6.31	4.03

[a] Expression levels were measured in *S. meliloti* strain 1021 and a 1021 RctA$^-$ mutant.

[b] Activity is indicated as specific β-glucuronidase activity (nmol/min/mg of protein). Standard deviations were calculated from at least three independent experiments.

[c] Relative activities are expressed as ratios of specific β-glucuronidase activity in the given backgrounds with respect to the corresponding 1021 wild-type background.

these genes were able to replace the *R. etli rctA* gene in silencing the conjugal transfer of the pRetCFN42d plasmid.

***S. meliloti rctA* gene represses conjugal transfer of the symbiotic plasmid pSymA.** As shown above, pSymA of *S. meliloti* also seems to contain a functional *rctA* copy, the SMa1323 gene. To test whether this gene has any influence in the repression of conjugal transfer of *S. meliloti* pSymA, an SMa1323 mutant derivative of strain 1021 was constructed, and pSymA transfer was tested in a mating with *A. tumefaciens* GMI9023 as the recipient. An *S. meliloti* 1021 derivative carrying pSymA tagged with an Sm/Spc cassette in the *otsA* gene was used as the donor in a control mating. The *rctA* mutation generated increments of *S. meliloti* pSymA transfer frequencies under laboratory conditions from undetectable to 1×10^{-5}, suggesting strong similarities in the regulation of pSym conjugal transfer between *S. meliloti* and *R. etli*. Furthermore, we also tested whether the *rctA* mutation in pSymA affects the expression of the conjugal transfer genes *traA1* and *traC*. Similar to the case for *R. etli*, a *rctA* mutation in *S. meliloti* resulted in enhanced expression of both Dtr genes (Table 5), which also correlates with the effect of this mutation on pSym transfer. Thus, the symbiotic megaplasmids from both species, *R. etli* and *S. meliloti*, share not only similar conjugal transfer genetic organizations but also similar genetic regulatory systems that limit their transfer under standard laboratory conditions.

DISCUSSION

The ability to nodulate leguminous plants provides rhizobia with the capacity to exploit an exclusive ecological niche and therefore with significant advantages over a strictly saprophytic lifestyle. Thus, it seems reasonable to think that the gain of the genetic information necessary to nodulate a specific host should be a very important event in the evolution of these soil bacteria. Genetic and ecological studies have shown that symbiotic plasmids do transfer in soil, as evidenced by the presence of similar pSyms in different genomic backgrounds (21, 36). These studies suggest the occurrence of horizontal transfer during the diversification of natural populations of rhizobia. Indeed, typical conjugal transfer genes have been identified in most pSym genomes sequenced so far.

Several symbiotic plasmids of *R. leguminosarum*, such as pRL1JI (33) and pRL5JI (8) of *R. leguminosarum* bv. viciae, pSym5 of *R. leguminosarum* bv. trifolii (29), and pRP2JI (37) of

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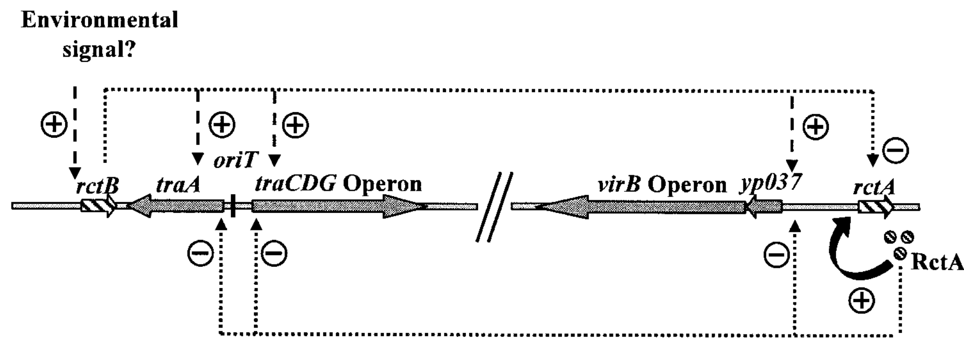

FIG. 4. Proposed model for conjugal transfer regulation of the *R. etli* symbiotic plasmid. +, activation; −, repression or inhibition. The lower part shows the normal status, i.e., the status under unfavorable conditions for conjugation (laboratory media), with high levels of RctA expression maintaining the silencing of conjugal transfer genes. When optimal environmental conditions for plasmid conjugation are found (upper part), the expression of *rctB* is enhanced; the *rctB* gene product interferes with *rctA* gene expression or RctA activity, relieving the expression of conjugal transfer genes so that plasmid conjugation can proceed.

R. leguminosarum bv. phaseoli, have been shown to be self-transmissible. However, for most rhizobial symbiotic plasmids, significant conjugal transfer does not occur under laboratory conditions, with transfer frequencies ranging from very low to undetectable.

In the particular case of *R. etli*, the transfer of pRetCFN42d has always been regarded as a cointegration phenomenon with the resident self-transmissible plasmid pRetCFN42a (10, 12, 57). Based on this, some authors suggested that the putative *oriT* and *tra* genes identified in the pRetCFN42d genome could be nonfunctional and viewed as evolutionary relics (22).

The demonstration of the functionality of the pRetCFN42d *mob* region and the identification of the yp028 gene as a possible activator of pRetCFN42d conjugative transfer provided the first strong evidence that this plasmid is self-transmissible (45). These findings led us to hypothesize the existence of a transfer regulation system that might repress the conjugal transfer functions under laboratory conditions. Here we have identified the *rctA* gene as an essential element of a regulatory system silencing the conjugative transfer of the *R. etli* symbiotic plasmid in standard laboratory media. Correlating with this, *rctA* is also required to maintain low expression levels of conjugal transfer genes in this plasmid. In contrast to that of transfer genes, the expression of *rctA* under laboratory conditions is high and seems to be positively autoregulated, as deduced from the low *rctA* expression levels observed in a *rctA* mutant. The putative *rctA* gene product shows no sequence homology to other proteins of known function. However, an analysis of the secondary and tertiary structures indicated that the predicted *rctA* gene product belongs to the winged-helix DNA-binding protein subfamily, which includes a number of both activators and repressors of gene transcription (18, 51). Altogether, the data suggest that the *rctA* gene product directly represses the transcription of transfer genes to prevent plasmid conjugation under nonfavorable conditions. The fact that the pRetCFN42d *rctA* mutant derivatives show self-transmissibility at high frequencies indicates that pSym conjugation can only be achieved under conditions leading to inactivation of the *rctA* gene product or to a reduction of *rctA* gene expression. This seems to be a requirement to obtain enhanced expression of the conjugal transfer genes *traA* and *traCDG* and the putative *virB*-like genes encoding a likely Mpf system. The *rctB* gene plays an important role in this regulatory process, as *rctA* expression is greatly reduced under conditions where *rctB* expression is enhanced, thereby leading to enhanced expression of transfer genes. Since *rctA* seems to autoregulate its own expression, it is not possible to anticipate whether the *rctB* product directly represses *rctA* transcription or interferes with the activity of the *rctA* gene product. There are two plausible mechanisms to explain the operation of this system. One of these entails binding of the RctA protein to its respective operators; this binding might be antagonized by protein-protein interactions with RctB. Alternatively, RctB might also bind to DNA, hindering the repression mediated by RctA. Resolution between these alternatives must await the purification of these proteins for use in binding assays in vitro.

Nonetheless, the data accumulated allow us to propose a working model for the regulation of *R. etli* pSym conjugative transfer (Fig. 4). We hypothesize that under unfavorable conditions for conjugation, like those present in the laboratory, the high expression of *rctA* allows the cell to maintain low expression levels of conjugal transfer genes so that efficient plasmid conjugation cannot proceed. Under favorable yet unknown conditions, the expression of *rctB* would be enhanced through an unknown mechanism, thereby leading to a reduction of *rctA* expression and/or activity which would result in the relief of expression of conjugal transfer functions and the transfer of pSym.

The regulatory network controlling pRetCFN42d conjugal transfer is probably very different from those described for other symbiotic and nonsymbiotic rhizobial plasmids, where plasmid transfer seems to be a quorum sensing-dependent phenomenon (15, 30, 57). This assumption is supported by the novel and important roles of the *rctA* and *rctB* genes in the control of pRetCFN42d conjugation, as described in this work, together with the absence of putative quorum-sensing gene homologs in pRetCFN42d (22).

We have also shown that conjugative transfer of the *S. meliloti* 1.35-Mbp pSymA is subject to *rctA*-dependent repression under laboratory conditions, by a mechanism likely similar to that found for pRetCFN42d. As for the *R. etli* pSym plasmid, our data provide strong evidence that the *S. meliloti* megaplasmid pSymA is self-conjugative but that this property is only expressed under certain conditions.

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Since *rctA* is functional in both plasmids, it seems reasonable to think that both pSyms may share other elements of the conjugation regulatory network. However, we have been unable to identify an *rctB* homolog within the pSymA sequence, which suggests that the derepression of conjugal transfer genes in both plasmids may be achieved through different regulatory cascades or in response to different environmental conditions.

The *rctA* gene does not seem to be exclusive to rhizobial Sym plasmids. The *A. tumefaciens* pAtC58 plasmid also contains an *rctA* gene, and we have shown that this gene, when expressed from a heterologous promoter, is able to replace the *R. etli* allele in inhibiting pRetCFN42d conjugative transfer. However, unlike the *R. etli* and *S. meliloti* pSyms, pAtC58 was reported to conjugate under laboratory conditions (13, 53), in spite of harboring a *rctA* gene. It is possible that *rctA* is not expressed in *A. tumefaciens*, or more likely, the RctA proteins are not able to cause a complete repression of plasmid conjugation in the *Agrobacterium* background. Evidence favoring the latter possibility comes from the facts that *R. etli* RctA was not fully able to repress the conjugation of the pRetCFN42d Tn*5*.1 derivative in the GMI9023 background, whereas the product of the *A. tumefaciens rctA* allele was able to totally repress the conjugation of pRetCFN42d *rctA* mutants in *R. etli* cells.

We have also identified a homolog of *rctA* within the genome of the biodegradative bacterium *Mesorhizobium* sp. strain BNC1, where it is also located divergently transcribed from a hypothetical *virB*-like operon. According to the unfinished genome sequence, this bacterium carries a single replicon and no extrachromosomal elements. Thus, it is possible that the *rctA* and *virB*-like genes found in this genome belong to a conjugative element integrated in the chromosome.

The presence of a system for the repression of conjugal transfer genes might represent a widespread regulatory system for conjugative elements within the *Rhizobiales* order as an alternative to the better-known quorum sensing-dependent regulation described for several rhizobial and agrobacterial plasmids. The existence of an active system to silence the expression of plasmid conjugal transfer genes suggests the importance of achieving lateral spread only under the most favorable conditions. Uncovering additional elements in this regulatory network will probably be the best way to identify the optimal conditions for pSym transfer in nature, a key aspect to fully understand the ecology and evolution of this group of symbiotic bacteria.

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Transcriptional Interference and Repression Modulate the Conjugative Ability of the Symbiotic Plasmid of *Rhizobium etli*▽†

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Bacteria of the order *Rhizobiales* are able to establish nitrogen-fixing symbioses with legumes. Commonly, genes for symbiosis are harbored on large symbiotic plasmids. Although the transfer of symbiotic plasmids is commonly detected in nature, there are few experimentally characterized examples. In *Rhizobium etli*, the product of *rctA* inhibits the conjugation of the symbiotic plasmid by reducing the transcription of the *virB* operon. *rctA* is transcribed divergently from this operon, and its product is predicted to have a DNA binding domain. In the present study, using DNase I footprinting and binding assays, we demonstrated the specific binding of RctA to the *virB* operon promoter. A 9-bp motif in the spacer region of this promoter (the *rctA* binding motif box) and the presence of a functional −10 region were critical elements for RctA binding. Transcriptional fusion analyses revealed that the elimination of either element provoked a relief of RctA-mediated repression. These data support a model in which RctA inhibits the access of the RNA polymerase to the *virB* promoter. Interestingly, *rctA* expression levels were modulated by transcriptional interference from transcripts emanating from the *virB* promoter. This phenomenon adds another level of regulation for this system, thus revealing a novel mechanism of plasmid transfer regulation in the *Rhizobiales*.

The ability to establish nitrogen-fixing symbioses is prevalent in bacteria of the order *Rhizobiales*. Commonly, most of the genes needed to establish symbiosis are either harbored on the so-called symbiotic plasmids (pSyms) or restricted to symbiosis islands (SI) located on the bacterial chromosome. As befits a trait that confers niche extension, there is evidence for the mobility of these genomic compartments. Indeed, sequence analyses of pSyms, including pRetCFN42d of *Rhizobium etli* (17), pNGR234a of *Rhizobium* sp. strain NGR234 (13), and pSymA of *Sinorhizobium meliloti* (1, 15), as well as of the SI of *Bradyrhizobium japonicum* (23, 18) and *Mesorhizobium loti* (22, 43), have lead to the identification of conjugation-related genes, mainly the *virB1*-to-*virB11* and *traA-traCDG* systems, carried by these elements. Moreover, a common feature of these genetic compartments is that the GC contents of these elements differ significantly from those of the rest of the genomes. These data suggest that these gene clusters originated out of, and were transmitted to, other genetic systems. It is likely that these compartments may still be prone to lateral transfer.

Evidence for the movement of pSyms among naturally occurring rhizobial populations has been inferred through phylogenetic and/or population genetics analyses of a variety of systems (45, 38). The transfer of SI, initially detected in field experiments investigating the SI of *M. loti* (41), was recently demonstrated for the SI of *B. japonicum* (16). Direct experimental evidence for lateral transfers has also been obtained, albeit such transfers have been found to occur at various rates (ranging from 10^{-3} to 10^{-9} transconjugants per receptor cell) for the SI of *M. loti* (42), the pSym pNGR234a of *Rhizobium* sp. NGR234 (20), and pRL1JI, the pSym of *Rhizobium leguminosarum* bv. *viciae* (10). Conjugational transfer in these three systems is regulated in part by quorum sensing (10, 20, 31), a common strategy used by other rhizobial nonsymbiotic plasmids, such as pTi of *Agrobacterium tumefaciens* (3) and pRetCFN42a of *R. etli* CFN42 (44). Thus, although pSym and SI transfer is widely detectable in nature, there are few examples in which mobilization and its regulation have been experimentally characterized.

R. etli CFN42 is a gram-negative bacterium capable of establishing a nitrogen-fixing symbiosis with the common bean (*Phaseolus vulgaris*). It contains six plasmids, with sizes ranging from 184 to 642 kb. One of them, pRetCFN42d (371 kb), is the pSym. A sequence analysis revealed that this plasmid also possesses a full set of genes involved in conjugation, comprising genes for a *m*ating *p*air *f*ormation (Mpf) type IV secretion system (2) and a *D*NA *t*ransfer and *r*eplication system (Dtr) (24). The genes for the Mpf system are arranged as a *virB1*-to-*virB11* operon with the peculiarity of possessing an additional gene, yhd0053, prior to *virB1*. The genes for the Dtr system include a *traA* gene, a functional relaxase gene (29), and a *traCDG* operon featuring genes for two accessory proteins (*traC* and *traD*) and a conjugative coupling protein (*traG*); interestingly, quorum sensing-related genes are absent from this plasmid.

Even though the automobilization of a pSym under laboratory conditions has never been detected, pSym transfer through cointegration with pRetCFN42a, a different automo-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant feature(s)	Source or reference(s)
Strains		
S. meliloti		
1021	Wild-type strain; Sm^r	15
A. tumefaciens		
C58	Wild-type nopaline-resistant strain	46
R. etli		
CFN2001	CFN42 derivative lacking p42a and p42d	6
CFN2001 Tn5.C	CFN2001 derivative with pRetCFN42d harboring a Tn*5* insertion in an unmapped location	28
CFN2001 Tn5.2	CFN2001 derivative with pRetCFN42d; *rctA*::Tn*5*	28
CFN2001 Tn5.6	CFN2001 derivative with pRetCFN42d; *rctB*::Tn*5*	28
E. coli		
S17.1	*thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn*7*	39
DH5α	*supE44* Δ*lacU169* ϕ80d*lacZ*ΔM15 *hsdR171 recA1 endA1 gyrA96 thi-1 relA1*	19
BL21(DE3)/pLysS	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3) pLysS (CamR)	Novagen
Plasmids		
p53Gus	pBBR1MCS5 derivative with a *uidA* gene of pWM5 (pBBR1MCS5::*uidA*)	L. Girard
p53*virB*::Gus	Transcriptional fusion of *virB* promoter in p53Gus using fragment pVT	28
p53*rctA*::Gus	Transcriptional fusion of *rctA* promoter in p53Gus using fragment pVT	28
p53*virB-10m*::Gus	Transcriptional fusion of *virB* promoter in p53Gus using fragment pVT-*10m*	This work
p53*rctA-10m*::Gus	Transcriptional fusion of *rctA* promoter in p53Gus using fragment pVT-*10m*	This work
p53*virB-rbm*::Gus	Transcriptional fusion of *virB* promoter in p53Gus using fragment pVT-RBM	This work
p53*rctA-rbm*::Gus	Transcriptional fusion of *rctA* promoter in p53Gus using fragment pVT-RBM	This work
pTE::*rctB*	pTE3 with *rctB* cloned in front of the *trp* promoter	27
pTE::*rctA*	pTE3 with *rctA* cloned in front of the *trp* promoter	28
pCR2.1-TOPO	PCR direct-cloning vector	Invitrogen
pET-16B	Protein His tag fusion and expression vector	Novagen
pRK404	Broad-host-range vector; Tc^r	11, 36
pSSH01	pCR2.1-TOPO with fragment pVT cloned	This work
pSSH02	pCR2.1-TOPO with fragment pVT-RBM cloned	This work
pSSH03	pCR2.1-TOPO with fragment pVT-*10m* cloned	This work
pSSH04	pET-16B with *rctA* cloned in NdeI/BamHI region	This work
pSSH05	pSSH04 fused to pRK404	This work

bilizable plasmid regulated by quorum sensing, was observed previously (44). The natural cointegration of these two plasmids occurs at a relatively high frequency and is mediated by both site-specific and homologous recombination (6).

Our previous work suggested that the pSym has an intrinsic ability for conjugal transfer, independent of pRetCFN42a, although this ability is tightly repressed (27). By various genetic strategies, two genes that participate in the regulation of the pRetCFN42d conjugational transfer were identified previously (28). The first one, named *rctA* (for *r*egulation of *c*onjugal *t*ransfer), is transcribed divergently from the *virB* operon, and it was determined previously by transcriptional fusion analyses to be a repressor of the *virB* genes. Consistent with the possible role of *rctA*, an in silico analysis of the predicted sequence of the corresponding protein revealed the presence of a winged-helix DNA binding domain. The second gene found, *rctB*, is located downstream of *traA*, and it appears to act as an inhibitor of the repressor activity of *rctA*. Functional homologues of all these genes also exist on plasmids pAtC58 of *A. tumefaciens* (46) and pSme1021a of *S. meliloti* (15), indicating that this model also applies to these organisms (28). Interestingly, this system represents a different alternative for the regulation of conjugal transfer in the *Rhizobiales* in which tight control is achieved by two novel regulator proteins in a quorum sensing-independent manner.

In the present study, using electrophoretic mobility shift assays (EMSA), DNase I footprinting, and transcriptional fusions, we characterized the mechanism by which *rctA* represses *virB* operon transcription. Our data demonstrate the specific binding of RctA to DNA and identify the specific sequence to which RctA binds in order to exert its repressor activity. Moreover, our work reveals the occurrence of transcriptional interference between the *rctA* and *virB* transcriptional units, a mechanism that conceivably allows the fine-tuning of conjugational activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Rhizobium* strains were grown at 30°C in PY rich medium (26) or in Y minimal medium containing 10 mM succinate and 10 mM ammonium chloride (5). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. Antibiotics were added, when required, at the following concentrations (in micrograms per milliliter): carbenicillin, 100 (*E. coli*); chloramphenicol, 15 (*E. coli*); gentamicin, 15 (*R. etli*) or 30 (*E. coli*); kanamycin, 15 (*R. etli*) or 30 (*E. coli*); nalidixic acid, 20 (*R. etli*); spectinomycin, 100 (*E. coli*); and tetracycline, 5 (*R. etli*) or 10 (*E. coli*). For the detection of β-galactosidase activity on agar plates, 30 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyrano-

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TABLE 2. Oligonucleotides used in this work

Name	Sequence[a]	Location[b]
38Tl	5′ TCCCGCCACAGCTTC 3′	161542 RetlpSym
38Tu	5′ TGCCGATCTGCTTCAGC 3′	161142 RetlpSym
Pv35u	5′ TTAATCGCGCTTGTCATGTCA TTTA 3′	161256 RetlpSym
Pv10u	5′ CATTTAACTGTGTTATATACGGC AGTA 3′	161265 RetlpSym
Pv1u	5′ TATACGGCAGTATGAATGCGG GCAG 3′	161280 RetlpSym
rbml	5′ CCGTATATAAC***CACTGGCCC***TGA CATGACAAGC 3′	161286 RetlpSym
rbmu	5′ GCTTGTCATGTCA***GGGCCAGTG***G TTATATACGG 3′	161254 RetlpSym
10Ml	5′ TTCATACTGCCG***AGACAT***ACACA GTTAAAT 3′	161295 RetlpSym
10Mu	5′ ATTTAACTGTGT***ATGTCT***CGGCA GTATGAA 3′	161266 RetlpSym
38SmTu	5′ CACGCGCCAGAGCTTCTC 3′	721157 SmelpSymA
38SmTl	5′ TCCCGAGCTGCTTCAGCC 3′	721556 SmelpSymA
38AtTl	5′ ACCACCTCCAAAGCTTCTC 3′	160059 AtumpTA
38AtTu	5′ ATGCCGATCTGCTTGATGC 3′	159661 AtumpTA
RctAl	5′ *AGGAATA*CATATGACAAGCGCGA TTAAAACGC 3′	161262 RetlpSym
RctAu	5′ *AAA*GGATCCCACTAAAGGCCGAA AAATCAGTC 3′	160872 RetlpSym
38Race	5′ CAACGGATGGTCGAGGATCTC 3′	161182 RetlpSym
37Race	5′ CGGGATTGGAAAGGCATAGGA 3′	161487 RetlpSym

[a] Restriction sites are underlined; noncomplementary bases are in italics; noncomplementary bases used for mutation are in bold italics.

[b] The location is indicated by the first 5′ nucleotide and the replicon where the sequence is located. RetlpSym, pSym of *R. etli*; SmelpSym, pSymA of *S. meliloti*; AtumpTA, plasmid A of *A. tumefaciens*.

side) ml^{-1} was used. For fusion analyses, cells were grown until mid-exponential phase in minimal medium. β-Glucuronidase activities in 1-ml culture samples were measured with *p*-nitrophenyl glucuronide as the substrate (8) and normalized according to the cell protein concentration.

Microbiological and DNA manipulations. Plasmids were isolated with the AquaPlasmid kit (MultiTarget Pharmaceuticals, Salt Lake City, UT). Plasmid transfer from *E. coli* to *Rhizobium* was done by biparental mating by using *E. coli* S17.1 with the appropriate plasmid as a donor. *Rhizobium* plasmids were visualized by the Eckhardt procedure (12). Plasmid transformation of *E. coli* was done using $CaCl_2$-competent cells (33).

Recombinant-DNA techniques were carried out using standard procedures (33). The primers used for PCR amplification are shown in Table 2. PCR amplifications were carried out with *Pfu* DNA polymerase (Altaenzymes, Alberta, Canada) in a TC-312 thermocycler (Techgene, Burlington, NJ). The DNA amplification regime consisted of 30 cycles comprising 94°C for 1 min, 1 min at variable temperatures, and 72°C for 1 min. For all PCR products cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), 3′ A overhangs were added. For ligations, T4 polynucleotide ligase (Amersham Biosciences, Piscataway, NJ) was used.

Promoter mapping. To map the transcriptional start sites of *rctA* and *virB*, 5-ml cultures of the appropriate strains expressing either *rctA* (CFN 2001 Tn5.C and CFN 2001 Tn5.C/p53*rctA*::Gus) or *virB* (strains CFN 2001 Tn5.2 and CFN 2001 Tn5.C/p53*virB*::Gus) were grown in PY medium and RNA was isolated using the High Pure RNA isolation kit (Roche, Nutley, NJ). Transcription initiation sites were mapped with a kit for the rapid amplification of cDNA 5′ ends (version 2.0; Invitrogen, Carlsbad, CA) using oligonucleotides 38Race and 37Race (Table 2) for *rctA* and *virB*, respectively. The products were sequenced to identify the transcription start sites. Promoter regions were predicted based on the *R. etli* promoter consensus (30).

Plasmid construction. Fragment pVT, encompassing the whole regulatory region comprising the promoters of both *rctA* and the *virB* operon (see Fig. 1), was amplified using primers 38Tu and 38Tl and cloned into pCR2.1-TOPO, yielding plasmid pSSH01. The mutant *virB* promoters were constructed with overlapping mutagenic oligonucleotides (34) by using primer pairs 38Tu/rbml and 38Tl/rbmu for the pVT-derivative fragment pVT-RBM and 38Tu/10Ml and 38Tl/10Mu (Table 2) for the pVT-derivative fragment pVT-*10m*. Both fragments were cloned into pCR2.1-TOPO, generating plasmids pSSH02 and pSSH03, respectively.

To construct the β-glucuronidase transcriptional fusions with the mutant *virB* promoters, plasmids pSSH02 and pSSH03 were cut with XbaI and KpnI. The resulting fragments were cloned separately into p53Gus restricted with XbaI-KpnI, generating plasmids p53*virB-rbm*::Gus and p53*virB-10m*::Gus. To construct transcriptional fusions of these fragments with the *rctA* promoter, we repeated the same procedure but using SpeI and XhoI, yielding plasmids p53*rctA-rbm*::Gus and p53*rctA-10m*::Gus.

To generate an amino-terminally His-tagged RctA derivative, the *rctA* coding sequence was amplified using primers RctAl and RctAu (Table 2), which contain custom-made NdeI and BamHI sites, respectively. After digestion with the appropriate enzymes, the PCR product was ligated into pET16b (40), which was cut similarly, giving rise to plasmid pSSH04. For introduction into *R. etli*, pSSH04 was digested with BamHI and ligated with BamHI-restricted pRK404 (11, 36) to yield pSSH05. All constructs were verified by DNA sequencing.

Overproduction and purification of RctA in *E. coli*. For the overproduction of RctA, cells of *E. coli* BL21(DE3)/pLysS/pSSH04 were grown in 100 ml of Luria-Bertani medium at 30°C to an A_{620} of 0.4. At this point, 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) was added; cells were harvested 2 h later, and the cell pellet was resuspended in 5 ml of ice-cold extraction buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). Cells were broken by three cycles of thawing and freezing, followed by three passages through a French press (Thermo Spectronic Instruments, Rochester, NY). The extract was centrifuged at 10°C for 10 min at 7,800 × *g* to obtain the cell-free fraction. To purify His-tagged RctA, a 1-ml Ni^{2+} affinity column (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with extraction buffer containing 100 mM imidazole. Five milliliters of cell extract containing the His-tagged RctA was added to the column, the column was washed with the same buffer, and His-tagged RctA was batch eluted with extraction buffer containing 200 mM imidazole. Proteins were analyzed by sodium dodecyl sulfate–16.5% polyacrylamide gel electrophoresis as described previously (25, 35).

EMSA analyses. DNA regions were amplified by PCR using the following oligonucleotide pairs: for *R. etli* CFN42 genomic DNA, 38Tu/38Tl (fragment pVT), Pv35u/38Tl (fragment pV-38), Pv10u/38Tl (fragment pV-29), and Pv1u/38Tl (fragment pV-14); for *S. meliloti* 1021 genomic DNA, 38SmTu/38SmTl (fragment pVT-*Sm*); for *A. tumefaciens* C58 genomic DNA, 38AtTu/38AtTl (fragment pVT-*At*); and for purified pSSH02 and pSSH03, 38Tu/38Tl (fragments pVT-RBM and pVT-*10m*, respectively). Products were electrophoresed on a 1.5% agarose gel and purified by band slicing (4). Fragments were 5′ end labeled with [γ-^{33}P]ATP by using T4 polynucleotide kinase (USB Corporation, Cleveland, OH). Unincorporated ATP was removed by gel filtration using Centri-Sep spin columns (Applied Biosystems, Foster City, CA). Labeling efficiency was measured by liquid scintillation analysis using an LS6500 counter (Beckman Coulter, Fullerton, CA).

His-tagged RctA was incubated with the desired fragments for 30 min at room temperature in binding buffer (20 mM Tris-HCl [pH 8.5], 10% glycerol, 50 mM KCl, 3 mM $MgCl_2$, 0.5 mg of bovine serum albumin/ml). For competition assays, the unlabeled fragment was added to the binding reaction mixture and the mixture was incubated for 10 min prior to the addition of the labeled fragment. Binding reaction mixtures were electrophoresed on a 6% TB-EDTA (Tris base, 40 mM; boric acid, 40 mM; EDTA, 1 mM)–polyacrylamide gel at 60 V for 1.5 h. The gel was dried on top of a Whatman filter paper and autoradiographed.

DNase I protection assay. Fragment pVT was ^{32}P labeled at the 5′ end of the bottom strand. A probe concentration equivalent to about 100,000 cpm was preincubated at room temperature with increasing concentrations of His-tagged RctA in the same binding buffer used for EMSA analyses. After 20 min, 0.003 U of DNase I (Roche, Nutley, NJ) in dilution buffer (8 mM Tris-HCl [pH 7.9], 40 mM $MgSO_4$, 4 mM $CaCl_2$, 40 mM KCl, 2 mM EDTA [pH 8.0], 24% glycerol) was added to the mixture and the mixture was incubated at room temperature for 2 min. The reaction was stopped by adding 300 μl of stop solution (570 mM ammonium acetate, 80% ethanol, 50 μg of carrier tRNA ml^{-1}). The DNA was precipitated, dried, and dissolved in 8 μl of loading buffer (45 mM Tris-borate [pH 8.0], 1 mM EDTA, 80% formamide). Samples were denatured at 85°C for 5 min and resolved by electrophoresis through an 8% polyacrylamide sequencing gel. Gels were vacuum dried and visualized with a PhosphorImager (Molecular Dynamics). Sequencing reactions were included for size markers.

RESULTS

***rctA* and *virB* are transcribed from convergent promoters.** Given the close proximity of *rctA* and the *virB* operon, a prerequisite to understanding their relationship was to map their

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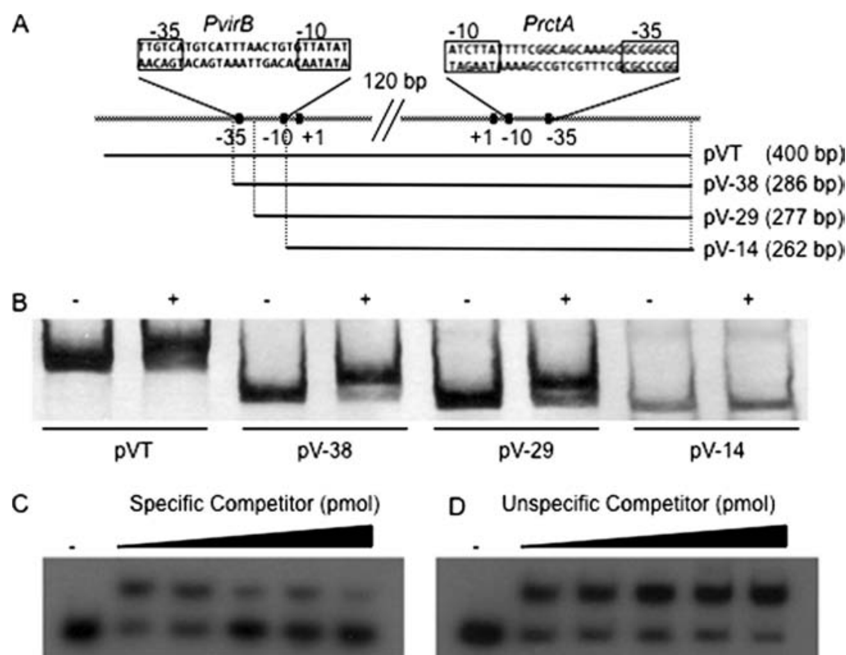

FIG. 1. Specific binding of RctA to the *virB* promoter region. (A) Scheme of the mapped promoters of *rctA* and the *virB* operon and the regions used in EMSA and transcriptional fusions. Fragment pVT encompasses the whole regulatory region comprising the promoters of both *rctA* and the *virB* operon, while fragments pV-38, pV-29, and pV-14 are shortened derivatives of pVT (the final number in each fragment designation indicates the terminal nucleotide, with respect to the transcriptional start site of *virB*). (B) Results of EMSA using fragments depicted in panel A. The DNA concentration was adjusted for homogeneity with the concentration (measured as counts per minute) of the probe. −, RctA not added; +, RctA added. The DNA/RctA molar ratio was always 1/1. (C and D) Results of competitive EMSA using fragment pV-38. The probed DNA concentration in each lane was 1.5 pmol; the RctA concentration was 1.5 pmol; specific (C) and nonspecific (D) unlabeled probes were added in increasing concentrations (0, 0.5, 1, 1.5, and 3 pmol). −, RctA not added.

promoters. To do so, we identified the start sites for each transcriptional unit by nucleotide sequencing of the products obtained in assays for the rapid amplification of cDNA 5′ ends (see Materials and Methods). To enhance the sensitivity of these assays, we employed mRNA for which the corresponding gene was transcribed either in *cis* (from the promoter in the pSym) or in *trans* (from the promoter cloned in the plasmid p53Gus). The methods produced identical results (data not shown) and allowed us to determine the transcription initiation sites and to predict the location of the promoter for each gene. As shown in Fig. 1A, the genes are transcribed from promoters facing each other (i.e., convergent promoters): while the *rctA* promoter is located within the coding sequence of yhd0053 (between nucleotides 55 and 84 of the predicted coding sequence), the *virB* operon promoter lies in the intergenic region between *rctA* and yhd0053 (the first gene of the *virB* operon). The location of the *virB* promoter was verified by mutagenesis (see below), while the position of the *rctA* promoter was further confirmed by deletion analysis (see Fig. S1 in the supplemental material).

RctA binds specifically to the *virB* operon promoter. As reported previously (28), RctA is predicted to have a winged-helix DNA binding domain (14, 32). This prediction suggests that the repressor activity of RctA may be due to direct binding to a regulatory region involved in the transcription of the *virB* operon. To test this hypothesis, we generated a His-tagged RctA derivative for use in EMSA (see Materials and Methods). To verify that this His-tagged derivative was functional in vivo, plasmid pSSH05 was introduced by conjugation into an *R. etli rctA* mutant derivative (see Materials and Methods) and the expression of both *rctA* and the *virB* operon was analyzed using the appropriate β-glucuronidase transcriptional fusions. As shown in Table 3, the His-tagged RctA derivative retained its biological activity, being able to complement an *rctA* mutant strain, as evidenced by the shutting off of the expression of the *virB* operon and the simultaneous activation of the transcription of *rctA*.

The His-tagged RctA derivative (molecular mass, 15.34 kDa) was purified to homogeneity by Ni affinity chromatography and then used to set up EMSA with different radiolabeled fragments encompassing the putative regulatory regions of *rctA* and the *virB* operon (Fig. 1A). For these experiments, fragment pVT encompassed the whole regulatory region comprising both the *rctA* and *virB* promoters, while fragments pV-38, pV-29, and pV-14 were shortened derivatives of pVT (the final number in each fragment designation indicates the terminal nucleotide, with respect to the transcriptional start site of *virB*). As shown in Fig. 1B, fragments pVT, pV-38, and pV-29 clearly formed RctA-dependent, retarded complexes, while the migration of fragment pV-14 was unaffected upon RctA addition. The main difference between the smallest retarded fragment (pV-29) and pV-14 was the absence in the latter of 15 bp, spanning part of the spacer region between the −10 and −35 boxes of the *virB* operon promoter (Fig. 1A). Thus, this region of the *virB* operon promoter is critical for the binding of RctA to DNA.

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TABLE 3. Activities of transcriptional fusions of *rctA* and *virB* promoters in different genetic backgrounds

Strain (relevant genotype)[a]	Sp act (nmol min^{-1} mg of protein^{-1})[b] from the indicated promoter in β-glucuronidase fusion with:					
	WT *virB*		*virB*- RBM		*virB-10m*	
	P*virB*	P*rctA*	P*virB*	P*rctA*	P*virB*	P*rctA*
CFN2001 Tn5.C (*rctA*$^+$ *rctB*$^+$)	59 ± 9	240 ± 18	220 ± 72	39 ± 13	ND	215 ± 9
CFN2001 Tn5.2 (*rctA rctB*$^+$)	257 ± 18	48 ± 15	237 ± 109	32 ± 22	ND	221 ± 16
CFN2001 Tn5.6 (*rctA*$^+$ *rctB*$^{++}$)	283 ± 58	67 ± 24	188 ± 56	29 ± 11	ND	229 ± 16
CFN2001 Tn5.2/pSSH05 [*rctA*$^+$(k) *rctB*$^+$]	87 ± 14	323 ± 55	NA	NA	NA	NA
CFN2001 (*rctA rctB*)	225 ± 57	33 ± 7	213 ± 68	55 ± 30	ND	220 ± 7
CFN2001/pTE*rctA* [*rctA*$^+$(tr) *rctB*]	2 ± 2	405 ± 100	247 ± 104	55 ± 30	ND	208 ± 24
CFN2001/pTE*rctB* [*rctA rctB*$^+$(tr)]	471 ± 51	4 ± 3	232 ± 77	24 ± 7	ND	230 ± 20

[a] +, wild-type expression; ++, unregulated expression; (tr), expression from the *trp* promoter; (k) expression from the pET16B promoter.
[b] Specific β-glucuronidase activities are expressed as means ± standard deviations of results from at least three independent experiments. WT *virB*, wild-type *virB* (on fragment pVT); *virB*-RBM, *virB* with a mutated RBM box (on fragment pVT-RBM); *virB-10m*, *virB* with a mutated −10 box (on fragment pVT-*10m*); ND, not determined; NA, not applicable.

To verify if the binding of RctA to fragment pV-38 was specific, competitive EMSA (see Materials and Methods) were set up. In these assays, when the binding of RctA to pV-38 was challenged by the prior addition of increasing amounts of unlabeled fragment pV-38 as a specific competitor, the amount of the retarded complex was reduced (Fig. 1C). In contrast, when an unlabeled competitor fragment from *S. meliloti* (a PCR product from nucleotides 1453158 to 1453424 of *S. meliloti* pSymB) was used, no decrease in the amount of retarded DNA complexes was seen (Fig. 1D).

These results clearly show that (i) RctA is able to bind specifically to DNA and (ii) a region located between the −10 and −35 regions of the *virB* promoter is needed for specific binding.

The binding of RctA depends on a conserved nucleotide sequence. As reported previously, *rctA* homologues negatively control the conjugative transfer of plasmids pAtC58 of *A. tumefaciens* and pSymA of *S. meliloti* (28). These *rctA* homologues can functionally substitute for *rctA* from *R. etli*. Therefore, it was reasonable to expect that RctA from *R. etli* should recognize similar sequences in *R. etli*, *A. tumefaciens*, and *S. meliloti*. Aiming to identify the nucleotides recognized by RctA, we made an alignment of the putative promoters of the *virB* operons from these three species. This alignment revealed the presence of nine nearly invariable nucleotides between the −10 and the −35 boxes of the *virB* promoter (Fig. 2A).

To verify that RctA of *R. etli* is able to bind to the *virB* promoter regions of *S. meliloti* and *A. tumefaciens*, we used specific oligonucleotides to amplify the equivalents of fragment pVT from each species (pVT-*Sm* and pVT-*At*, respectively) to use them in EMSA. As shown in Fig. 2B, retarded complexes with both fragments were found upon the addition of similar proportions of RctA from *R. etli*.

To demonstrate the role of the conserved nine base pairs in the binding of RctA to DNA, we constructed a mutant version of fragment pVT in which these nucleotides were changed from TTT AAC TGT to GGG CCA GTG, generating fragment pVT-RBM. When EMSA was performed with this fragment, RctA from *R. etli* was unable to bind, even upon the addition of an eightfold molar excess of RctA versus pVT-RBM (Fig. 2C). These results demonstrate that nucleotides within a conserved 9-bp sequence, termed the *rctA* binding

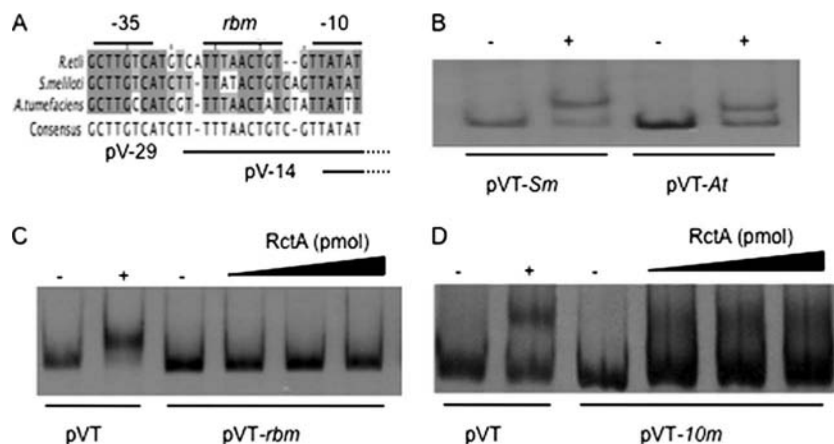

FIG. 2. The binding of RctA requires an RBM box and an active −10 region. (A) Multiple-sequence alignment of the *virB* promoters of three rhizobiales showing the nucleotide conservation that delineates the RBM box. Solid horizontal lines below the alignment mark the limits of regions pV-29 and pV-14 with reference to the *virB* promoter. (B) Results of EMSA using fragments pVT-*Sm* and pVT-*At*; the DNA concentration was 1 pmol. −, RctA not added; +, 2 pmol of RctA added. (C and D) Results of EMSA using fragments pVT-RBM and pVT-*10m*, respectively. The DNA concentration in each lane was 1 pmol; RctA was added in increasing concentrations (0, 2, 4, and 8 pmol). −, RctA not added; +, 2 pmol of RctA added.

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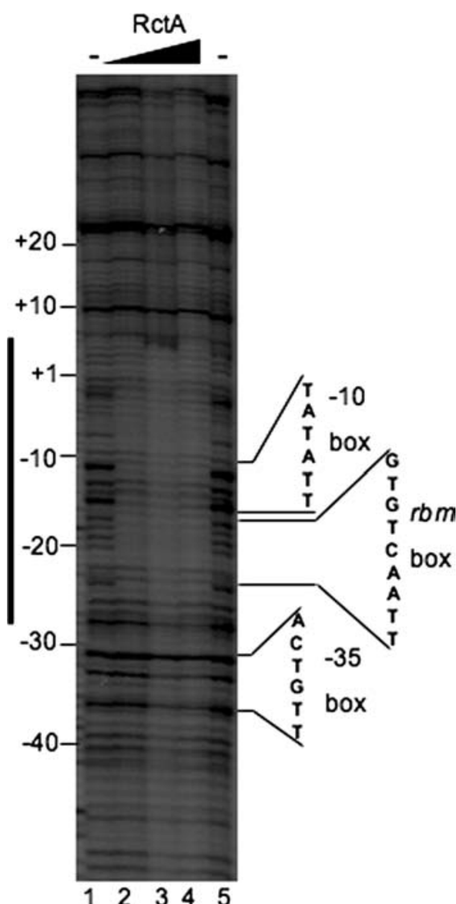

FIG. 3. DNase I protection of the *virB* operon regulatory region by RctA. Increasing amounts of His-tagged RctA were mixed with a ^{32}P-end-labeled DNA fragment corresponding to fragment pVT and treated with DNase I. Samples were subjected to electrophoresis on an 8% polyacrylamide sequencing gel. The −10 and −35 promoter sequences and the RBM box are indicated on the right; they were determined by running sequencing reactions with the same fragments in parallel (data not shown). The protected regions are indicated by a vertical black bar. The black triangle above the autoradiogram represents increasing amounts of RctA. Lanes: 1 and 5, DNA alone; 2 to 4, 2, 4, and 8 pmol of RctA, respectively. −, RctA not added.

motif (RBM) box, are required for the binding of RctA to DNA.

The binding of RctA protects a zone encompassing the RBM box and the −10 region. The identification of a motif in the spacer region of the *virB* promoter needed for the binding of RctA is fully consistent with the proposed role of this protein as a transcriptional repressor. To ascertain if the binding of RctA obliterates the access to other transcriptional elements, we performed a DNase I protection assay of the pVT fragment in the presence of increasing amounts of RctA (Fig. 3). Our results show a well-delineated protection zone in the *virB* promoter upon the addition of RctA, even at the lowest protein concentration tested. This region encompassed nucleotides −26 to +5 of the *virB* promoter region relative to the transcription start site and, as expected, included the RBM box (nucleotides −17 to −25). Interestingly, although the −35 box (nucleotides −32 to −37) was not within the protected region, the −10 box (nucleotides −10 to −15) of the promoter was significantly protected. This result further supports the idea that the interaction of RctA with the *virB* promoter may impair the transcription of the *virB* operon.

Moreover, the finding that the −10 region was also protected in the presence of RctA opens up the possibility that this sector is also needed for the binding of RctA. To test this possibility, we constructed a pVT mutant fragment in which the −10 region was changed from TTA TAT to AGA CAT. This mutant fragment (pVT-*10m*) was then used for EMSA in the presence of various amounts of RctA. As shown in Fig. 2D, only a scarce amount of retarded complexes with the pVT-*10m* fragment was seen, even at an eightfold molar excess of RctA versus DNA. Interestingly, the amount of these retarded complexes was not increased when larger amounts of RctA were added, suggesting that these complexes were unstable in vitro. Thus, these results indicate that the binding of RctA to DNA requires elements located both in the RBM box and in the −10 region.

The binding of RctA to the *virB* promoter represses *virB* operon transcription. Since fragment pVT harbors the promoters for both the *rctA* gene and the *virB* operon, the introduction of this fragment into a promoterless *uidA* reporter plasmid allows an evaluation of the expression of both promoters, depending on the orientation of the insert. To explore the functional consequences of the mutation in the RBM box for the expression of *virB* and *rctA*, we constructed two transcriptional fusions with the fragment pVT-RBM, one in the direction of the *rctA* promoter (p53*rctA-rbm*::Gus) and the other in direction of the *virB* operon promoter (p53*virB-rbm*::Gus).

The introduction of fusions with wild-type promoters into an otherwise wild-type background confirmed, as previously reported (28), low-level expression from the *virB* promoter but high-level expression from the *rctA* promoter (Table 3). In contrast, when RBM mutant fusions were introduced into a wild-type background, we found that the level of expression from the *virB* promoter was high but that expression from the *rctA* promoter was diminished (Table 3). The expression patterns obtained with these mutant fusions closely matched the one found with a wild-type-promoter fusion in an *rctA* mutant background (Table 3). In fact, the expression pattern seen for the RBM mutant fusions (a high expression level for the *virB* promoter and a low expression level for the *rctA* promoter) was maintained in backgrounds lacking *rctA* or overexpressing *rctB* (Table 3). Given the location of the RBM sequence and the inability of RBM mutant constructs to bind RctA, these results are fully consistent with the interpretation that the binding of RctA to the *virB* promoter represses the transcription of this operon.

The transcription of the *virB* operon interferes with *rctA* expression. It has been reported previously (28) that mutations in *rctA* have the interesting effect of provoking a reduction of *rctA* expression (Table 3). This effect was also seen under conditions that conceivably interfered with RctA function, such as the overexpression of RctB (Table 3). These observations were explained by invoking the hypothesis of positive autoregulation for this gene (28). However, the convergent organization of the *virB* and *rctA* promoters, coupled with the presence of a single RctA binding site far from the *rctA* promoter, raises the alternative possibility that transcription from

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the *virB* promoter interferes with *rctA* expression. In this view, the loss of the repressor (as in an *rctA* mutant) or the blocking of its activity (as in a strain overexpressing *rctB*) should allow transcription from the *virB* promoter, which may structurally interfere with expression from the *rctA* promoter.

These two hypotheses (positive autoregulation and transcriptional interference) can be distinguished by studying the expression patterns of both *rctA* and *virB* genes in a mutant affected in the −10 box of the *virB* promoter. According to the positive-autoregulation hypothesis, the loss of *virB* expression should have no effect on *rctA* expression, which would remain high in a wild-type background or low in either an *rctA* mutant strain or a strain overexpressing *rctB*. In contrast, according to the transcriptional-interference hypothesis, the loss of transcription from the *virB* promoter would provoke a high level of constitutive transcription from the *rctA* promoter. To discern between these alternatives, we constructed two transcriptional fusions with the fragment pVT-*10m*, one in the direction of the *rctA* promoter (p53*rctA-10m*::Gus) and the other in the direction of the *virB* operon promoter (p53*virB-10m*::Gus). As shown in Table 3, *virB* expression was completely abolished when this mutant fragment was used. Notably, high-level constitutive expression of *rctA* from this mutant fusion was observed even in an *rctA* mutant and in a strain overexpressing *rctB* (Table 3). Interestingly, high-level constitutive expression of *rctA* was observed despite the fact that in the *virB* −10 box mutant fragment (pVT-*10m*), RctA binding was severely reduced (Fig. 2D). These observations are fully consistent with the expectations of the transcriptional-interference model.

To further substantiate this point, all the transcriptional fusions were introduced into genetic backgrounds lacking the pSym and, hence, both *rctA* and *rctB* (Table 3). In one of these strains, *rctA* was supplied on a separate plasmid under the control of the strong tryptophan promoter, while in another *rctB* was overexpressed (Table 3). Interestingly, the high-level constitutive expression of *rctA* from the *virB* −10 box mutant gene-*rctA*::Gus fusion was maintained even under circumstances in which *rctA* expression should have increased or decreased (Table 3) according to the positive-autoregulation hypothesis. Thus, these results clearly reveal that the expression of *rctA* is modulated by transcriptional interference emanating from the *virB* promoter.

DISCUSSION

In this work, we have provided direct evidence for the role of RctA as a transcriptional repressor of conjugational transfer genes in *Rhizobium*, based on structural and functional data. Using site-specific mutagenesis and EMSA, we have identified a 9-nucleotide motif in the spacer sequence of the *virB* operon promoter that is required for specific binding, which we have named the RBM box. As shown by DNase I footprinting assays, the binding of RctA protects a region that encompasses not only the RBM box, but also the −10 region of the *virB* promoter. This protection pattern fully explains the fivefold reduction in *virB* expression previously observed, since RctA binding should hinder the access of the RNA polymerase to the *virB* promoter. The binding of RctA to the *virB* promoter regions of both *A. tumefaciens* pAtC58 and *S. meliloti* pSymA, as demonstrated here, indicates that the regulatory characteristics described here should extend to these homologous systems (28).

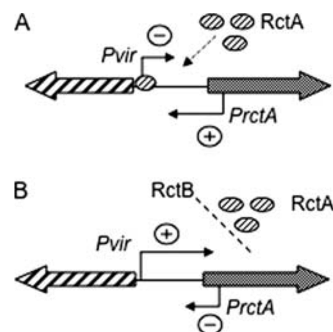

FIG. 4. Role of transcriptional interference in the regulation of the *virB* operon. (A) The *virB* operon promoter is blocked by the binding of RctA to the RBM box, repressing the expression of the promoter and allowing *rctA* transcription without interference. (B) RctB blocks RctA access to the RBM box, allowing *virB* operon transcription, which in turn interferes with *rctA* expression. +, activation; −, repression or inhibition.

Interestingly, although the RBM box is the main determinant for RctA binding, it is not the sole factor. As shown in Fig. 2D, the elimination of the promoter −10 box near the RBM box significantly reduced the binding of RctA. In this sense, the −10 box played an important role, albeit an ancillary one, in the binding of RctA. The requirement for a functional −10 box adjacent to the RBM box has important consequences for recognition. This requirement ensures that the binding of RctA should be targeted to active promoters. In this regard, it is germane to mention that two potential RBM boxes have been located, by sequence analysis, near the *traCDG* region. Only one of these boxes, the one that has a recognizable −10 box nearby, is bound by RctA (unpublished data).

A second important regulatory aspect that emerges from our data is the presence of transcriptional interference. As mentioned before, the convergent organization of the *virB* and *rctA* promoters generates the possibility of interference between them. Transcriptional interference has been defined as the suppressive influence of one transcriptional process, directly and in *cis*, on a second transcriptional process (37) and has been observed previously for artificially convergent promoters (21, 9) and bacteriophage promoters (7). As revealed by data from the transcriptional fusions, the *rctA-virB* region in *R. etli* shows all the hallmarks of transcriptional interference. When either RctA or the RBM box was absent, the transcription of the *virB* operon was activated, simultaneously reducing the transcription of the *rctA* promoter. Our data show that the reduction in *rctA* transcription was due most likely to transcriptional interference and not to autoregulation by RctA, as previously thought. Support for this conclusion comes from the fivefold increase in *rctA* transcriptional activity upon the elimination of transcription from the *virB* promoter. This effect was observed even in the absence of the whole pSym, thus ruling out any potential influence in *trans* as an explanation for this phenomenon.

The finding of transcriptional interference adds another level for the regulation of this system. Under conditions that limit conjugative transfer, the expression of the *virB* operon is

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repressed by the binding of RctA to the *virB* promoter; this binding provokes high-level expression of *rctA* due to the lack of transcriptional interference, thus ensuring tight repression of the system (Fig. 4A). Whenever RctA binding is diminished, *virB* expression is activated, thus ensuring the establishment of transcriptional interference with *rctA*. This last effect warrants the full expression of the conjugative system (Fig. 4B).

A critical aspect of this system relates to the conditions that allow the elimination of repression by RctA. Thus far, the activation of conjugation has been seen upon the inactivation of *rctA* or the overexpression of *rctB*. There are two common mechanisms that regulate the conjugational transfer of automobilizable plasmids, quorum sensing (3, 6) and peptide signaling (3), but neither of them seems to participate in pSym transfer in the absence of pRet42a (unpublished results). Future work will be devoted to identifying environmental signals that preclude RctA functioning and the detailed role of *rctB* in this process.

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RESULTADOS ADICIONALES

***yhd0053* forma parte del operón *virB*.**

Para poder hacer la caracterización de la regulación del operón *virB* es necesario establecer su estructura y localizar su promotor. Debido a que no hay antecedentes para la localización de un gen homólogo a *yhd0053* en un operón *virB* es necesario establecer si este gen forma parte del operón y están compartiendo un promotor.

Para determinar si el gen *yhd0053* forma parte de una unidad transcripcional con los genes *virB*, se realizó un experimento de RT-PCR (transcriptasa reversa seguida de PCR). Para éste fin, se utilizaron los oligonucleótidos RTV3 (ATGCGGGTTGAGCAGCGAGTGG) y RT37V (GCCAGTGCATGGGCTATCCGATAAGTC), cuyo producto abarcaba los últimos 147 nucleótidos de *yhd0053* y los primeros 103 de *virB1.* Tras la amplificación a partir de RNA se obtuvo un producto de 250 pb (Fig. 5) cuya identidad fue confirmada por digestión y secuenciación automática.

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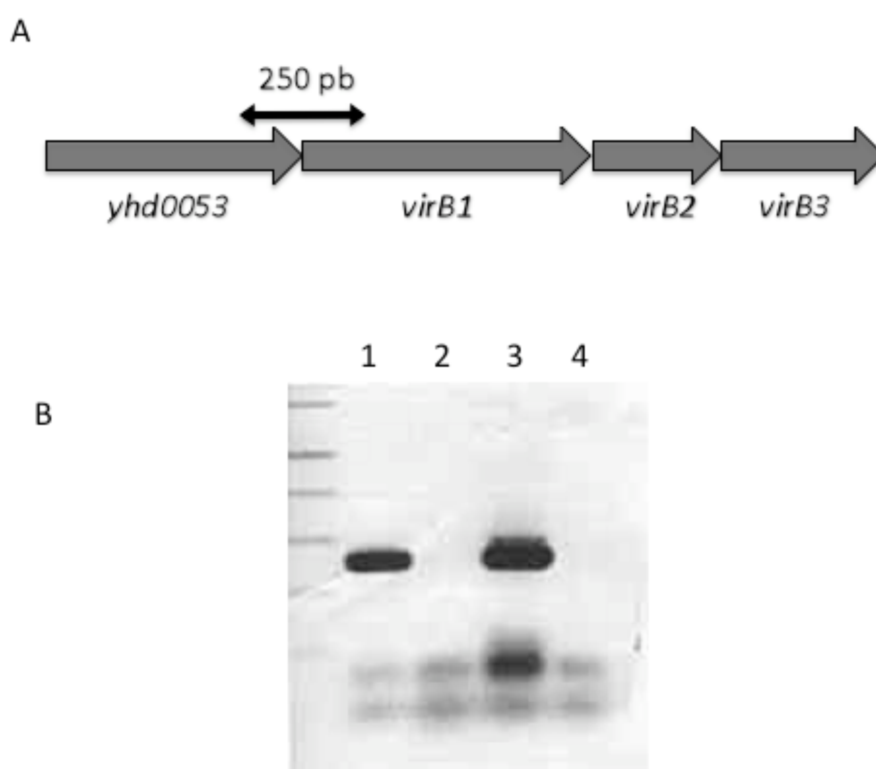

Figura 5.- RT-PCR de *yhd0053* y *virB1*. A.- Localización del producto esperado. B.- RT-PCR *yhd005-virB1*- 1.-PCR a partir de DNA genómico; 2.-PCR a partir de RNA purificado; 3.-RT-PCR; RT-PCR sin templado.

Si bien este resultado confirma la existencia de un transcrito que contiene tanto a *yhd0053* como a *virB1*, para confirmar que forman parte de un operón se requiere demostrar que comparten un mismo promotor. Con este objetivo, a la cepa CFN2001 p42d::Tn5.6 (en la que la movilización del pSym es constitutiva) se le introdujo por recombinación una inserción de 4.5 kb entre el promotor de *yhd0053* y *virB1* generando la cepa CFN2001 p42d::Tn5.6 *yhd0053*ins (Fig. 6).

Los ensayos de conjugación bacteriana mostraron que mientras que la cepa CFN2001 p42d::Tn5.6 tenía una frecuencia conjugativa del pSym de $1.31x10^{-3} \pm .2.5x10^{-7}$, la cepa CFN2001 p42d::Tn5.6 *yhd0053*ins era incapaz de movilizar el plásmido. Este resultado es compatible con el del experimento de RT-PCR y nos permite afirmar que el gene *yhd0053* forma parte del operón *virB*.

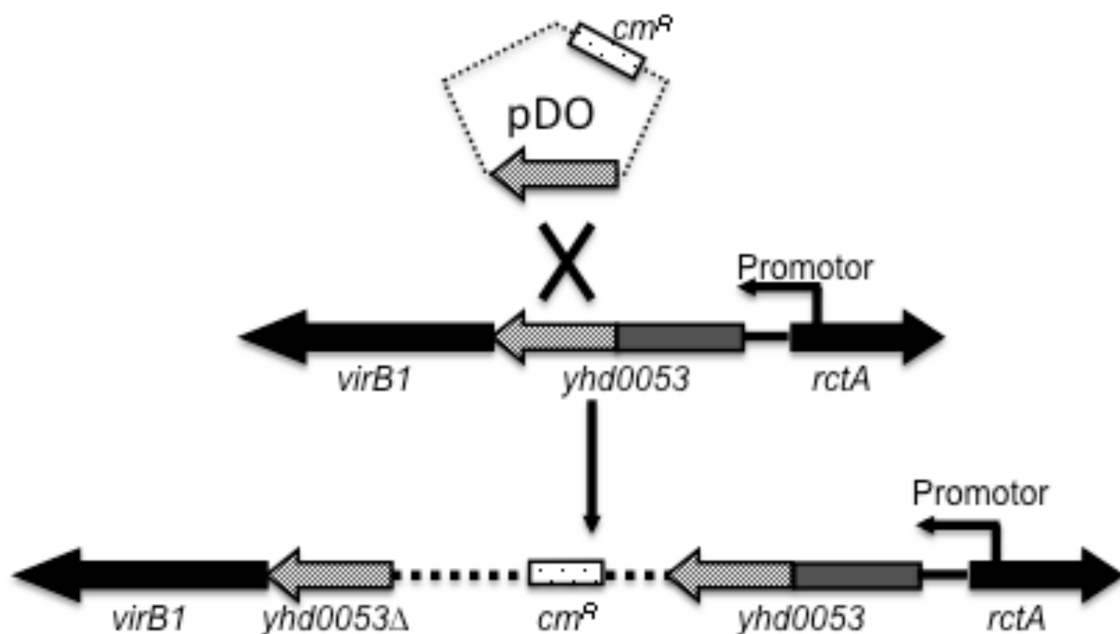

Figura 6.- Estrategia para construir la cepa CFN2001 p42d::Tn5.6 *yhd0053*ins. El plásmido pDO no replica en *Rhizobium etli* por lo que tras su conjugación la resistencia a *cm* solo puede mantenerse si el plásmido se cointegró por recombinación. Ningún promotor del plásmido quedó en dirección del operón *virB*.

El gen *yhd0053* se encuentra conservado también el *A. tumefaciens y S. meliloti* y aunque se predice que posee un dominio de unión a ATP/GTP, no es posible asignarle una función. Sin embargo al formar parte del operón *virB* es factible que juegue un papel en la transferencia conjugativa. Para probar esta posibilidad, a partir de la cepa CFN2001 p42d::Tn5.6 construimos la mutante CFN2001 p42d::Tn5.6 *yhd0053*Δ que posee una deleción, en fase, de 300 nucleótidos en *yhd0053*. La mutación fue introducida por doble recombinación utilizando un plásmido no replicable en *Rhizobium* (pDO) que tenía clonada la versión deletada de *yhd0053* (generada por medio de PCR).

Al realizar ensayos de conjugación encontramos que la cepa mutante mostraba una disminución de un orden de magnitud con respecto a la CFN2001 p42d::Tn5.6 ($3.00x10^{-4}$± $.1.2x10^{-8}$ VS. $1.31x10^{-3}$± $.2.5x10^{-7}$). Estos resultados muestran que si bien *yhd0053* tiene un papel durante la transferencia conjugativa, éste no es esencial.

RctA reconoce una caja *rbm* en la región de los genes *traACDG*

Debido a que los experimentos con fusiones transcripcionales revelaron que *rctA* también participa en la regulación de los genes *traACDG*, es factible predecir la presencia de una caja *rbm* en la región regulatoria de éstos. Una búsqueda manual nos permitió identificar dos secuencias candidatas (Fig. 7A). La primera, *rbm*A, se localiza en la región intergénica entre *traA* y el operón *traCDG* y la segunda, *rbm*B, se localiza entre los nucleótidos 62 y 70 de la región codificante de *traC.* Por medio de ensayos de retardo, con diferentes recortes de la zona, logramos determinar que RctA es capaz de unirse a la región *tra* del pSym y que esta unión depende de la secuencia *rbm*A (Fig. 7 A y B).

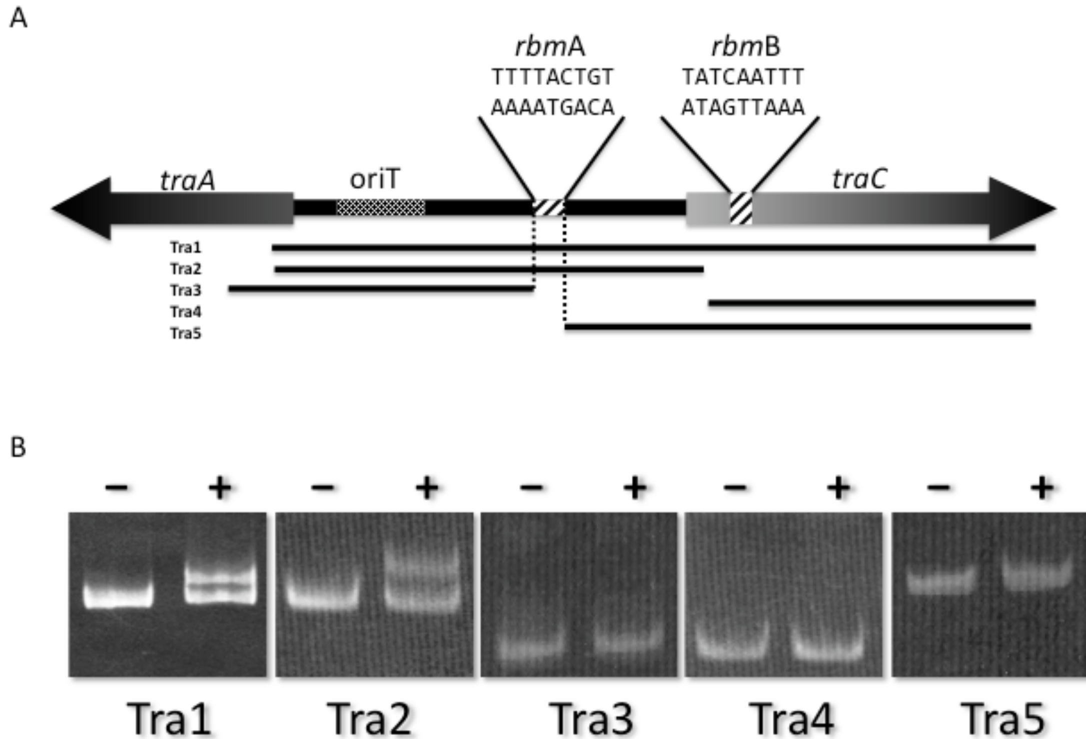

Figura 7.- Localización de las probables cajas *rbm* en la región *tra*. A. Esquema de la región con la ubicación de cada caja *rbm* y las regiones utilizadas en los ensayos de retardo. B. Ensayos de retardo con las regiones definidas en A. – Sin RctA; + Con RctA. Las concentraciones de DNA y RctA se ajustaron 1:1. El DNA se tiñó con bromuro de etidio.

Hacia un consenso de la caja *rbm*

Una vez que se confirmó que la caja *rbm* de los genes *tra* es reconocida y unida por RctA se añadió su secuencia al alineamiento de cajas *rbm.* Esto nos permitió mejorar el consenso calculado para los nueve pares de bases que lo componen, e identificar seis bases que son invariables (Fig. 8A). Curiosamente, cuando comparamos con el consenso la secuencia de la secuencia *rbm*B, que no es reconocida por RctA, observamos que era idéntica a la caja *rbm* de los gene *vir* de *A. tumefaciens*, que sí es reconocida por RctA. La explicación para esta observación podría radicar en la secuencia adyacente a ésta. Como se recordará, tenemos evidencia experimental que demuestra que la caja -10 del promotor de *virB* es necesaria para el reconocimiento por RctA. Para tratar de encontrar una secuencia consenso dentro de las cajas -10 adyacentes a las cajas *rbm* repetimos los alineamientos incluyendo la secuencia adyacente a éstas. Los resultados de

este alineamiento muestran un consenso con cuatro bases invariables dentro de las probables cajas -10 adyacentes a las cajas *rbm* (Fig. 8B). Cuando comparamos con este consenso la secuencia de la caja *rbm*B ninguna de las bases adyacentes coincidía con este consenso, reforzando la hipótesis de que estas son importante para el reconocimiento por RctA.

A

R. etli virB	TTTAACTGT
R. etli traAC	TTTTACTGT
S. meliloti virB	TTATACTGT
A. tumefaciens virB	TTTAACTAT
Consenso	TTTWACTGT

B

R. etli virB	TTTAACTGT--GTTATAT
R. etli traAC	TTTTACTGT--TTTATTC
S. meliloti virB	TTATACTGTCAGTTATAT
A. tumefaciens virB	TTTAACTATCTATTATTT
Consenso	TTTWACTGT---TTATWT

Figura 8.- Alineamientos de las cajas *rbm* identificadas en Rhizobiaceas. A. Alineamiento de las cajas *rbm*. B. Alineamiento añadiendo las probables cajas -10. Los alineamientos se generaron utilizando Clustal X.

DISCUSIÓN

En este trabajo hemos iniciado la caracterización del mecanismo que regula la transcripción del operón *virB* del plásmido simbiótico de *R. etli*. Se han identificado dos genes como reguladores de este sistema:

El gen *rctA* actúa como represor del operón *virB* al unirse RctA, su producto, al promotor de *virB* e impedir la unión de la RNA polimerasa. El gen *rctB*, por medio de un mecanismo aún no determinado, es un antagonista de la actividad de *rctA* (Fig. 9). Adicionalmente, hemos encontrado que la transcripción emanada del promotor de *virB* ejerce un efecto inhibitorio sobre el promotor de *rctA* por medio de interferencia transcripcional. Ésto explica por qué en cepas mutantes afectadas en *rctA* disminuye la actividad del promotor de *rctA*. Este fenómeno constituye un nuevo nivel de regulación para la transferencia conjugativa que permite la expresión ininterrumpida de los genes necesarios para la formación del par conjugativo.

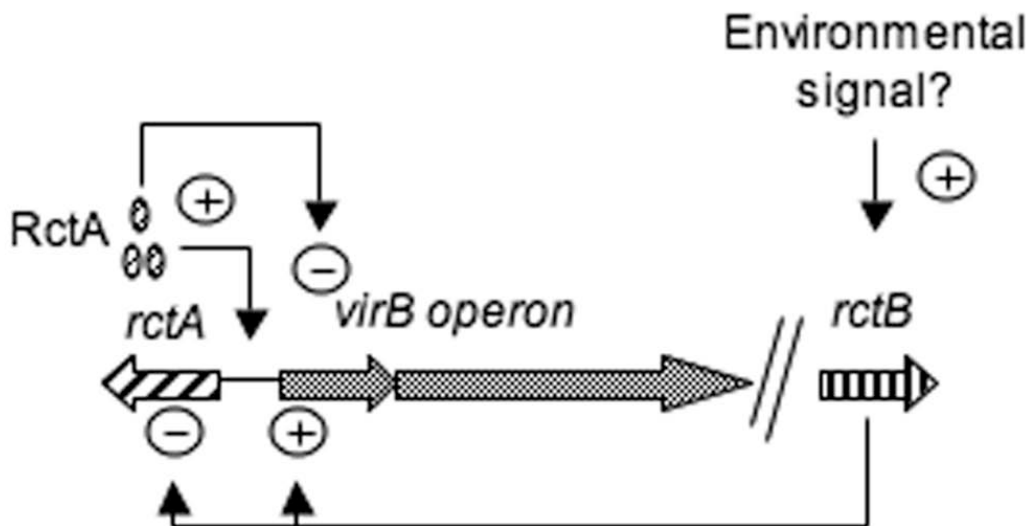

Figura 8.- Esquema de la regulación transcripcional del operón *virB*.

La especificidad de la unión RctA-DNA depende de la presencia de una región conservada de nueve nucleótidos que hemos denominado caja *rbm* (**R**cta **b**inding **m**otif) y también de la presencia de una caja -10 adyacente a ésta. Nuestros alineamientos han resaltado 6 bases invariables de la caja *rbm* y 5 de la caja -10 que podrían ser claves en el reconocimiento de DNA por RctA. Estos alineamientos fueron construidos utilizando las cajas *rbm* que son reconocidas por RctA: las del promotor del operón *virB* de *A. tumefaciens, S. meliloti y R. etli* y la de la región *tra* de *R. etli*. Como se

mencionó en la introducción, los plásmidos donde se localizaron en cada especie están relacionados filogenéticamente. Esto nos permite predecir la existencia de dos cajas *rbm* más: una localizada en la región *tra* de *A. tumefaciens* y otra en la de *S. meliloti*. Para incrementar la exactitud de nuestro consenso es necesario ubicarlas, comprobar experimentalmente si RctA es capaz de unirse a éstas y, si es así, incluirlas en los alineamientos. El consenso fortalecido, obtenido a partir de estos, nos permitiría elegir las bases más conservadas para que, mediante experimentos de mutación dirigida y ensayos de retardo en movilidad electroforética, podamos determinar experimentalmente los nucleótidos críticos para la unión de RctA al DNA. Adicionalmente, el consenso robustecido puede ser utilizado para hacer búsquedas en el genoma de las tres Rhizobiaceas en que se ha encontrado *rctA*, para tratar de localizar otros genes que se encuentren bajo la regulación de este gen. Si esta búsqueda se complementa con estudios de expresión global en *R. etli* (microarreglos), comparando el perfil de expresión de una cepa silvestre con el de una cepa *rctA*$^-$ y una sobreexpresando *rctB*, podríamos definir el regulón de *rctA*. Esta aproximación nos podría revelar nuevos genes involucrados en la conjugación y otras vías relacionadas que también se encuentren bajo el control de *rctA* y *rctB*.

Uno de los aspectos mas novedosos del sistema regulatorio del operón *virB* es la participación de la interferencia transcripcional como un nivel adicional de regulación del operón *virB*. Existen pocos ejemplos en procariontes en que este sistema juega un papel principal en un sistema regulatorio, por lo que sería importante caracterizar finamente este fenómeno.

Se han propuesto varios modelos para explicar la interferencia transcripcional entre promotores convergentes (Shearwin *et al.,* 2005). El modelo de competencia entre promotores, en el cual la ocupación de un promotor por el complejo de la RNA polimerasa estorbaría la unión de otro complejo de RNA polimerasa en un promotor adyacente, no se ajusta al sistema *rctA/virB* dada la distancia (120 pb) que existe entre los promotores. El modelo de obstrucción del promotor, en el cual el paso de un complejo de RNA polimerasa en elongación limita el acceso a otro promotor, es también poco probable de aplicarse a este caso. Este modelo requiere que los promotores involucrados tengan fuerza desigual, de tal forma que el promotor fuerte

ocluye el débil, sin embargo nuestros datos de actividad transcripcional relativa demostraron que ambos promotores tienen fuerza similar.

Existen otros dos modelos que se ajustan mejor a las características de nuestro sistema. Uno de ellos es el modelo de colisión (Crampton *et al.,* 2006), en que dos complejos de RNA polimerasa en elongación convergen en un punto entre los dos promotores, ocasionando el desprendimiento de uno o ambos complejos. El otro modelo es conocido como "pato sentado" o "pichón" (Callen *et al.,* 2004), en el que un complejo de RNA polimerasa de elongación rápida converge con un complejo que aún no inicia la elongación, provocando nuevamente el desprendimiento de uno o ambos complejos. Para poder discernir entre estos dos modelos, será necesario llevar a cabo experimentos de transcripción *in vitro* en ausencia de RctA. Bajo el modelo de colisión detectaríamos transcritos completos del promotor de *virB* y transcritos abortados del promotor de *rctA*. Bajo el modelo del "pichón" no detectaríamos el transcrito proveniente del promotor de *rctA*, mientras que sí detectaríamos el transcrito sintetizado a partir del operón *virB*. Existe poca evidencia experimental de los mecanismos de interferencia transcripcional por lo que los resultados de estos experimentos serían una importante contribución.

Si bien hemos podido establecer el papel que juega *rctB* como un antagonista de la actividad represora de *rctA,* no hemos determinado el mecanismo a través del cual cumple su función y la falta de dominios conservados dificulta hacer una predicción. Entre otras opciones podría actuar como un anti-represor uniéndose directamente a RctA, de tal forma que desplace o impida la unión de la proteína a la caja *rbm* o a través de una proteína aún no identificada. Por medio de los ensayos de retardo se puede determinar si RctB interfiere con el mecanismo de unión a DNA de RctA. Para realizar estos experimentos se debe contar con RctB purificada en estado soluble. Intentos previos de sobreexpresar y purificar la proteína solo han logrado obtenerla en cuerpos de inclusión. La dificultad para obtener la proteína soluble puede radicar en la cantidad de residuos de cisteínas (7) que posee RctB. Estos aminoácidos son capaces de formar enlaces disulfuro que deben establecerse entre residuos específicos para lograr un plegado correcto de las proteínas. Existen alternativas que pueden explorarse para sobre-

expresar proteínas con alto número de cisteínas en forma soluble. Entre estas se encuentran el uso de plásmidos que expresan chaperonas o cepas con mutaciones en la tioredoxin-reductasa (*trxB*) y la glutatión reductasa (*gor*) que optimizan la formación de los enlaces disulfuro en el citoplasma. En caso de no lograr la expresión en forma soluble existen otras alternativas que nos permitirían definir si RctA interactúa con RctB u otras proteínas como ensayos de doble híbrido y de copurificación.

Para poder obtener una visión integral de la regulación de la transferencia conjugativa del plásmido simbiótico aún quedan dos tareas pendientes. La primera consiste en determinar cómo reprime RctA la transcripción de *traA* y del operón *traCDG*. Dada la actividad de unión a DNA de RctA y de la estructura de la caja *rbm* (que requiere la presencia de la caja -10 de un promotor) podemos predecir que RctA reprime los genes *tra* impidiendo el acceso de la RNA polimerasa a sus promotores. A pesar de que en este caso es poco probable que también se dé un fenómeno de interferencia transcripcional, este sistema presenta una peculiaridad que lo vuelve muy interesante. Como se recordará, hemos logrado identificar una caja *rbm* funcional en la región intergénica de *traA* y *traCDG*. Adicionalmente nuestros experimentos también descartaron la presencia de una segunda caja en la región codificante de *traA* y *traC*. Esto hace muy probable que los dos promotores de los genes *tra* sean regulados a través de una sola caja *rbm*. Experimentos de cromatografía de exclusión de masa con RctA nativa (datos no mostrados) parecen indicar que RctA es capaz de formar multímeros que podrían abarcar dos promotores, aún en caso de que estos estuvieran muy separados. Anteriormente se intentó localizar los promotores de los genes *tra* por medio de 5´RACE sin éxito, debido a que la actividad transcripcional de estos genes es mucho más baja que la del operón *virB*. Esto puede resolverse clonando los promotores en un plásmido de alto número de copias y determinar el sitio de inicio de la transcripción en *trans.* Alternativamente, podrían realizarse experimentos de *primer extension*, que si bien tienen menor resolución, requieren niveles de expresión menos altos. Otra explicación para los bajos niveles de expresión observados en estos genes es la autorepresión que éstos ejercen al llevar a cabo su función conjugativa. Para superar este problema bastaría con construir mutantes en la relaxasa o

las proteínas accesorias. Además de que constituirían una importante herramienta en la determinación del esquema regulatorio de los genes *tra*, podrían proporcionarnos niveles de expresión aceptables para los experimentos de mapeo de promotores.

La segunda tarea pendiente es la determinación de la condición efectora del sistema. Hasta ahora la única forma de inducir la transferencia conjugativa del pSym es por medio de la inhabilitación de *rctA* o de la sobre-expresión artificial de *rctB.* El que el sistema no se haya logrado inducir en condiciones de laboratorio no es de extrañarse, dado que la mayoría de los sistemas de conjugación se encuentran estrictamente reprimidos. Dado que tenemos identificado el gen antirepresor es necesario identificar las condiciones bajo las cuales incrementa su transcripción.

Basados en los sistemas regulatorios descritos para otros plásmidos, hemos realizado experimentos buscando determinar esta señal activadora. Desafortunadamente, no se ha observado activación de los genes conjugativos mediante el uso de medios condicionados ni a alta densidad celular, condiciones óptimas en sistemas regulados via quorum sensing o feromonas. Tampoco en presencia de exudados de raíz y de frijol, útiles en la inducción por moléculas señal de plantas. Inclusive, se han probado otras condiciones que son más afines a la inducción de fagos, como variaciones en temperatura, pH y salinidad. También se han intentado identificar posibles condicionantes ambientales, variando las fuentes de carbono y nitrógeno y con la adición de metales pesados como el Zinc y el Cobalto, todas ellas sin éxito. Las opciones son amplias, sin embargo, sería interesante ampliar nuestros experimentos desde el punto de vista de las interacciones ecológicas, por ejemplo se podría probar condiciones de biofilm bajo las que se sabe que se potencia la conjugación (Molin y Tolker-Nielsen, 2003). E incluso ir mas allá, probando diferentes combinaciones como un biofilm sobre una raíz de frijol. La gama de probabilidades es tan amplia que podríamos probar decenas de condiciones sin lograr encontrar la que induce la conjugación. Fernández-Lopez y colaboradores desarrollaron un ensayo de bioluminiscencia dependiente de conjugación con el cual probaron más de 12, 000 compuestos biológicamente activos para encontrar inhibidores de la transferencia conjugativa (Férnandez-López et al., 2005). Este ensayo podría

adecuarse a nuestro sistema con facilidad y nos permitiría ampliar la cantidad y variedad de compuestos aumentando la probabilidad de encontrar un compuesto inductor de la conjugación del pSym.

Existe otro medio de obtener información biológica relevante sobre el sistema. Se han aislado otras cepas de *Rhizobium* que poseen plásmidos con sistemas conjugativos relacionados con el del p42d (Susana Brom, comunicación personal). La adición de estas cepas a nuestros ensayos permitirían maximizar la probabilidad de encontrar un inductor del sistema que sin lugar a dudas estaría relacionado con el que induce al p42d.

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