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Inestabilidad del Fenotipo Simbiótico de *Rhizobium phaseoli*: Un Enfoque en el Estudio del Genoma de *Rhizobium*.

Tesis que para obtener el grado de Doctora en Investigación Biomédica Básica presenta Gloria Soberón Chávez

Cuernavaca, Morelos. 1986.

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A José y a nuestros hijos

Inés y Adrían.

INDICE

Presentación.

La presente tesis engloba diversos enfoques en la investigación de la inestabilidad del fenotipo simbiótico de R. phaseoli; presento estos enfoques escritos en forma de artículos de investigación ya que mi inteción es que se publique en alguna revista del área, así mismo a manera de introducción al trabajo de investigación presento un artículo de divulgación que publiqué en "Investigación y Ciencia". Algunos de los datos que no están incluídos en ningún artículo, los presento - al final como un capítulo más.

En la discusión final resalto algunos puntos generales de mi trabajo, ya que la discusión de los datos está incluído en cada artículo; y propongo algunas estratégias futuras de mi investigación.

Mecanismo de nodulación de las leguminosas

La investigación genética de Rhizobium *ha permitido avanzar en el estudio del mecanismo de nodulación de las leguminosas y de la dinámica de la población de estas bacterias en el suelo*

Gloria Soberón

Todos los seres vivos requieren una fuente nitrogenada para crecer, ya que los principales componentes celulares, como las proteínas y los ácidos nucleicos, contienen nitrógeno. Varían, sin embargo, los compuestos nitrogenados que los distintos organismos son capaces de asimilar; las plantas utilizan compuestos inorgánicos, como amonio (NH_4^+) o nitrato (NO_3^-); los animales superiores, además de amonio, requieren compuestos orgánicos, como aminoácidos o bases nitrogenadas.

En última instancia, todos los compuestos nitrogenados que los seres vivos utilizan provienen del nitrógeno molecular (N_2), que representa el 80 por ciento de los gases que forman la atmósfera terrestre. Los únicos organismos capaces de utilizar directamente este elemento son las bacterias fijadoras de nitrógeno. Los demás dependen del metabolismo de estos microorganismos para tener una fuente nitrogenada. Dichas bacterias asimilan el nitrógeno molecular por medio de la enzima nitrogenasa, proteína que cataliza la conversión de nitrógeno en amonio hidrolizando adenosín trifosfato (ATP).

En la mayoría de los habitats del planeta, la productividad biológica está limitada por la disponibilidad de una fuente nitrogenada, de modo que si se aumentara la fijación de nitrógeno aumentaría la biomasa del ecosistema. La disponibilidad de amonio o nitrato en el suelo condiciona también el crecimiento de muchas plantas cultivadas por el hombre, que han de fertilizarse para aumentar los rendimientos.

Los fertilizantes nitrogenados que se utilizan en la agricultura se sintetizan a partir de nitrógeno atmosférico; este proceso requiere de alta presión y temperatura, que se alcanzan a expensas de combustibles derivados del petróleo. Si los requerimientos de las plantas pudieran satisfacerse directamente por la fijación biológica de nitrógeno habría un ahorro de combustible y se sustituirían los recursos naturales no renovables por la energía derivada, en última instancia, del sol.

El poder acoplar la fijación de nitrógeno con su asimilación por plantas de importancia económica es un objetivo muy prometedor, aunque todavía lejano. Un enfoque que puede proporcionar información valiosa para provocar la interrelación entre las bacterias fijadoras de nitrógeno y plantas cultivadas es el de estudiar las soluciones que se han seleccionado en la naturaleza. Hasta el momento, la relación mejor estudiada es la simbiosis que se establece entre bacterias del género *Rhizobium* y algunas leguminosas.

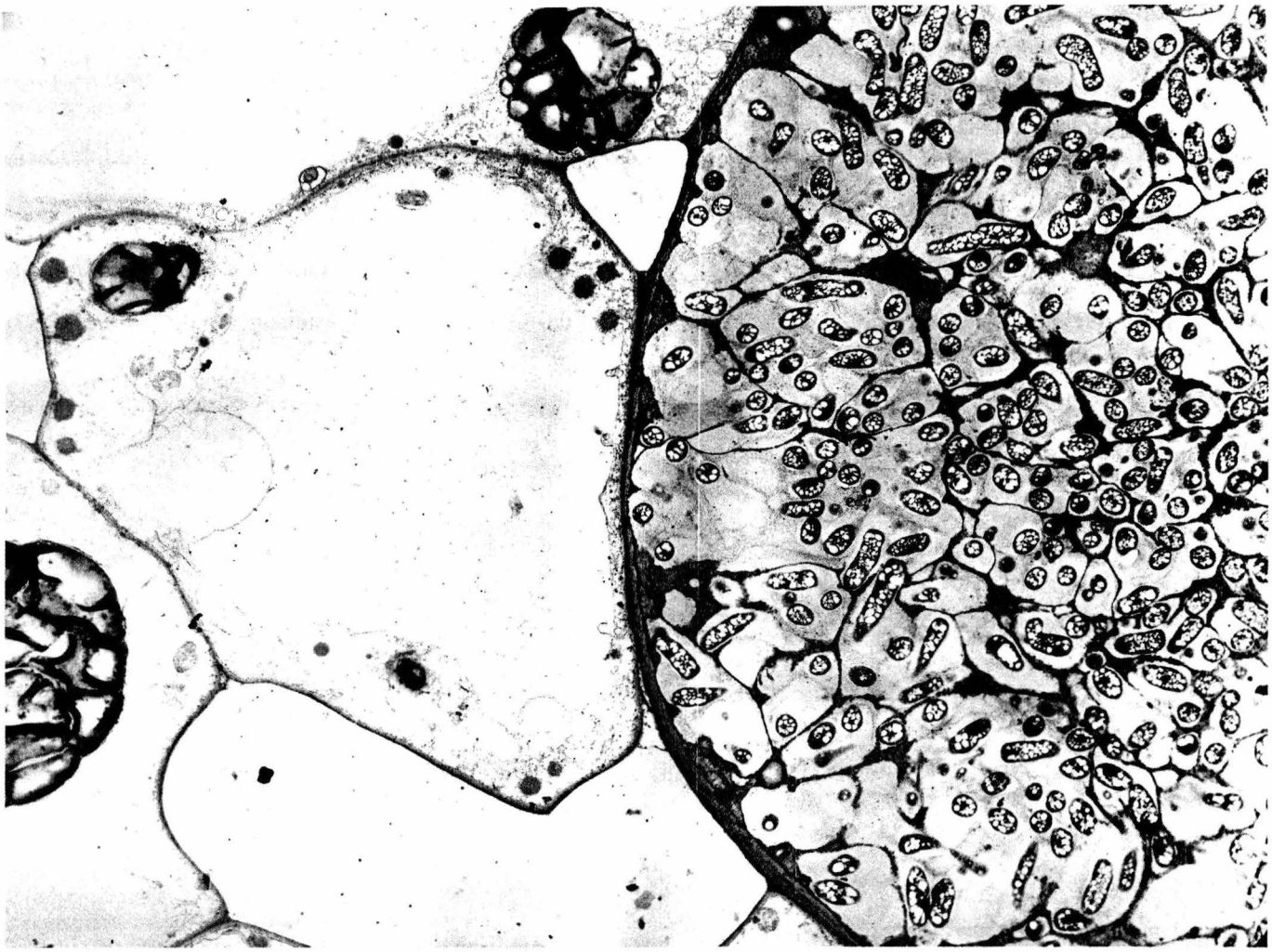
Las bacterias del género *Rhizobium* son habitantes naturales del suelo que infectan las raíces de ciertas leguminosas. Al llevarse a cabo esta infección, se desarrollan unas estructuras denominadas nódulos, donde las bacterias se dividen y fijan nitrógeno. La especie a la que pertenece un cierto *Rhizobium* se define por la especie de la planta que nodula y en la que es capaz de fijar nitrógeno; así pues, la bacteria que se asocia con el guisante se conoce como *Rhizobium leguminosarum* y, la que lo hace con el trébol, *Rhizobium trifolii*, etcétera.

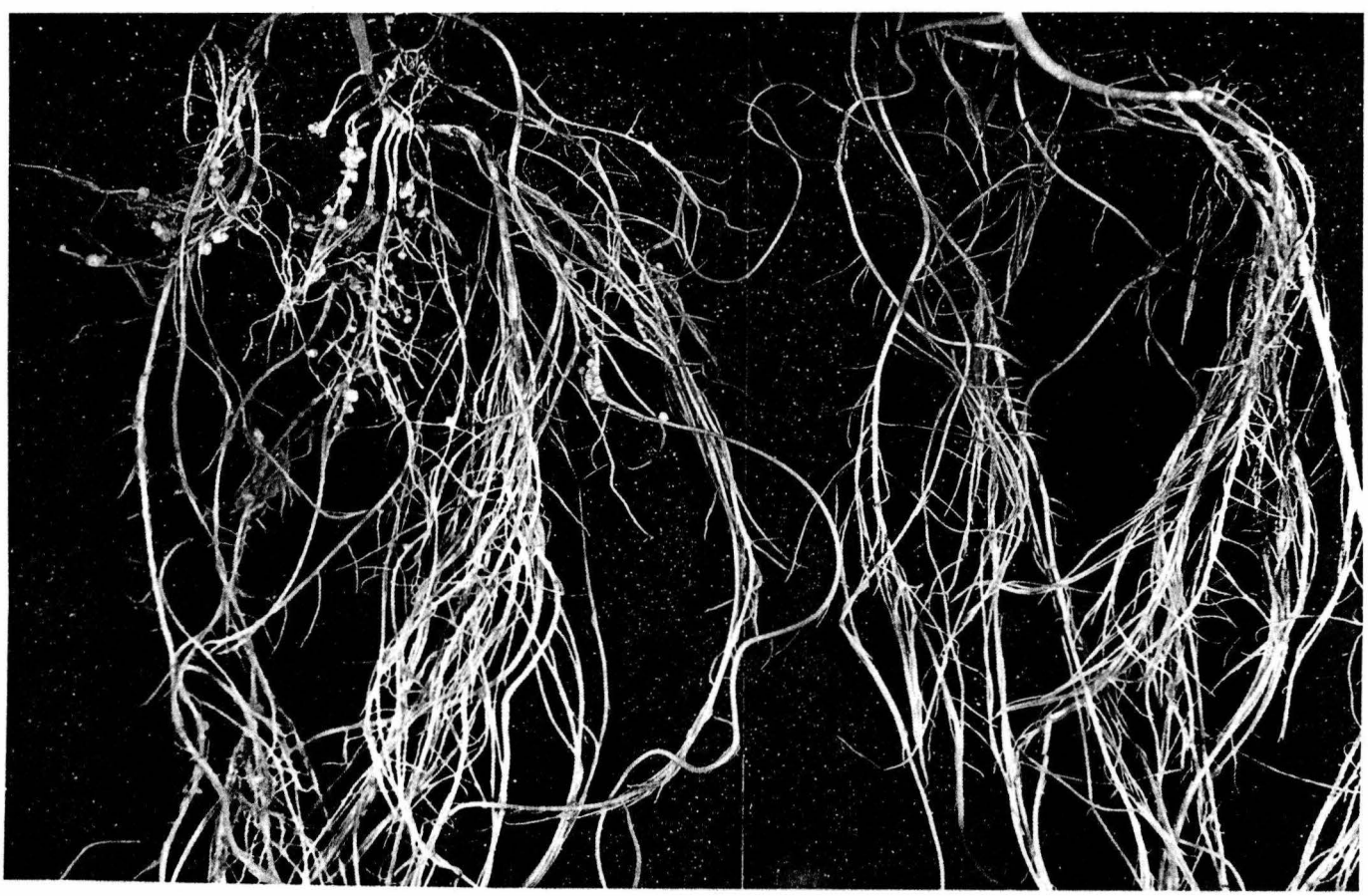
El nódulo está formado por tejido de la planta. Algunas de las células que lo constituyen contienen bacterias englobadas en membranas dentro de su citoplasma; estas bacterias atraviesan un proceso de diferenciación y es precisamente la forma diferenciada (bacteroide) la que fija el nitrógeno. El bacteroide es mayor que la bacteria, posee una forma irregular y se muestra más sensible a cambios de presión osmótica.

El nódulo viene a ser, pues, un órgano producto de la simbiosis, encargado de la fijación de nitrógeno y la asimilación de este elemento. A los nódulos llegan carbohidratos producto de la fotosíntesis; de ellos se exporta a las estructuras superiores de la planta el nitrógeno fijado por el bacteroide y transformado en el citoplasma de la célula vegetal en compuestos orgánicos nitrogenados, como aminoácidos o derivados de bases nitrogenadas. De este modo, el nitrógeno requerido para el crecimiento de la planta lo aporta la bacteria; la leguminosa proporciona la energía necesaria para la actividad de la nitrogenasa y para el mantenimiento del nódulo.

Cuando se cultiva una leguminosa, el número de *Rhizobia* presentes en el suelo capaces de nodularla aumenta en varios órdenes de magnitud, lo que muestra que el *Rhizobium* específico se beneficia de la presencia de su planta hospedadora; se desconoce, sin embargo, el mecanismo responsable de que esto suceda. Es poco probable que las bacterias que forman los nódulos y se convierten en bacteroides aumenten en número, pues el bacteroide es mucho más frágil que la bacteria y no parece regresar a su forma original. Otra expli-

1. LA INTERACCION DE *RHIZOBIUM PHASEOLI* con la raíz de fríjol tiene como resultado la formación de nódulos en los que se lleva a cabo la fijación del nitrógeno (*abajo*). Arriba se ofrece una micrografía obtenida por Federico Sánchez, del Centro de Investigación sobre Fijación de Nitrógeno, en la que se pueden apreciar una célula infectada que contiene bacteroides y células no infectadas. La raíz del fríjol de la izquierda, abajo, corresponde a una planta inoculada con una cepa silvestre de *R. phaseoli*; a la derecha, otra planta inoculada con la misma cepa después de un tratamiento que elimina plásmidos.





cación podría ser que la fracción de la población de *Rhizobium* que no infecta a la planta se dividiera en presencia de ésta; pero esto podría producir que se amplificara la información más ineficaz para la simbiosis, pues serían las bacterias que no forman nódulos las que se desarrollarían. Como se verá más adelante, este fenómeno podría justificarse atendiendo a la reordenación de los plásmidos, que portan la información de *Rhizobium* que participa en la simbiosis con la leguminosa.

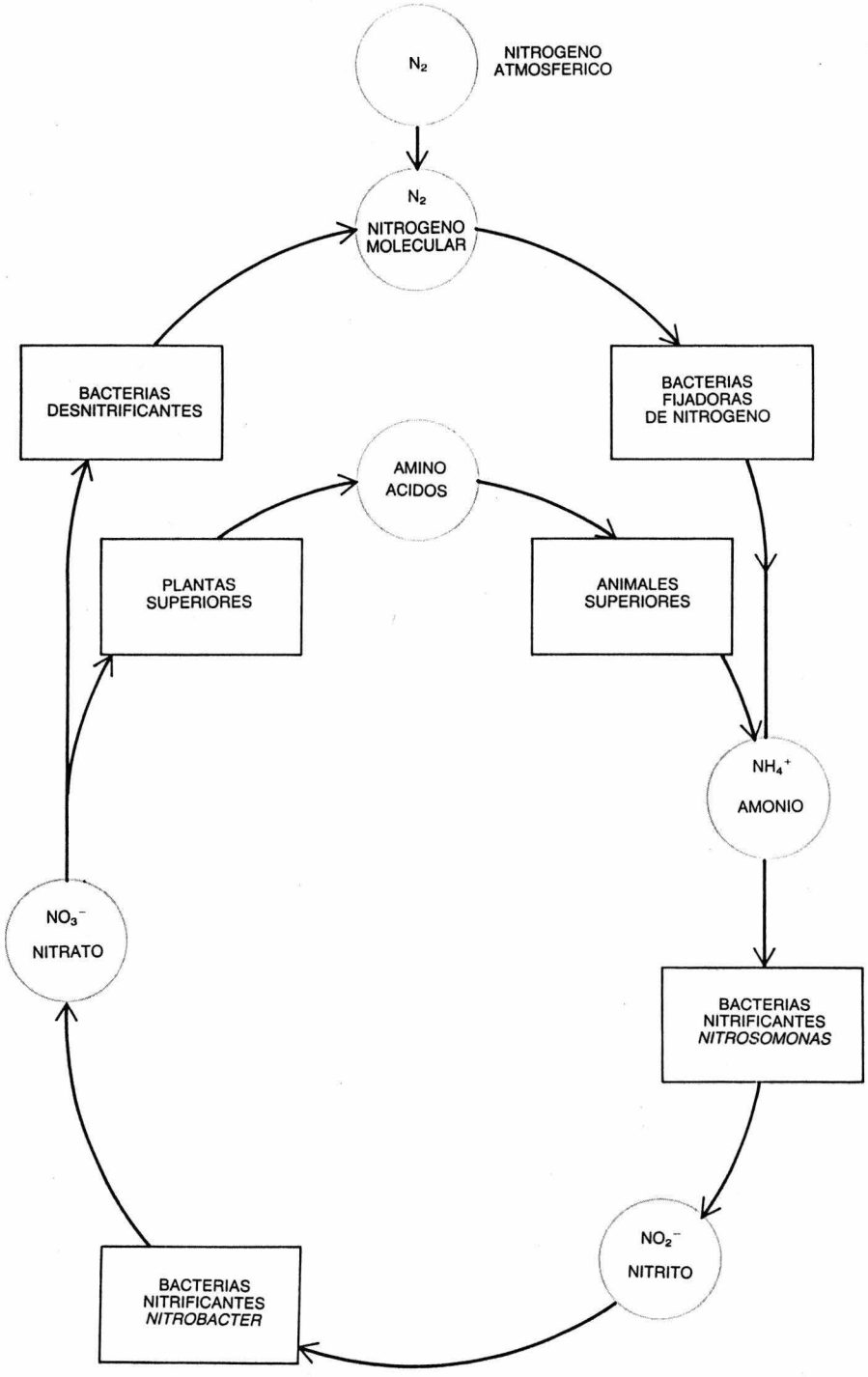
Según se ha establecido, la información genética que determina que *Rhizobium* sea capaz de infectar específicamente una leguminosa y fijar nitrógeno en asociación con ella está codificada en ciertas moléculas de ADN extracromosómico conocidas como plásmidos.

Los primeros datos al respecto los aportó, en 1967, S. Higashi, de la Universidad de Tokyo. Demostró que la especificidad para nodular trébol se transfería por conjugación de *R. trifolii* a una cepa que originalmente nodulaba fríjol (*R. phaseoli*). Ya que la transferencia por conjugación es una característica propia de los plásmidos, este resultado demuestra que la especificidad para la nodulación está codificada en una de estas moléculas.

Más tarde se demostró la transferencia de la especificidad de nodulación entre varias especies de *Rhizobium* como *R. leguminosarum* y *R. trifolii*. Posteriormente, el grupo de R. A. Schilperrort, de la Universidad holandesa de Leiden, puso de manifiesto la transferencia de la capacidad de nodular trébol, aunque no de fijar nitrógeno, de *Rhizobium trifolii* a una cepa de *Agrobacterium tumefaciens*, que naturalmente no nodula ninguna planta. Recientemente en el Centro de Investigación sobre Fijación de Nitrógeno de la Universidad Nacional Autónoma de México, el grupo de M. Mejías y el nuestro encontramos que *Agrobacterium tumefaciens* es capaz de nodular fríjol y de fijar nitrógeno en estos nódulos al heredar ciertos plásmidos provenientes de *Rhizobium phaseoli*.

Otro enfoque experimental usado es el de tratar *Rhizobium* con agentes que eliminan plásmidos. Se ve cómo se pierde la capacidad de nodular y de fijar nitrógeno. Gracias a este enfoque W. Zurkowski, de la Universidad Nacional de Australia, ha identificado qué plásmidos llevan información para la nodulación de varias cepas de *R. trifolii*.

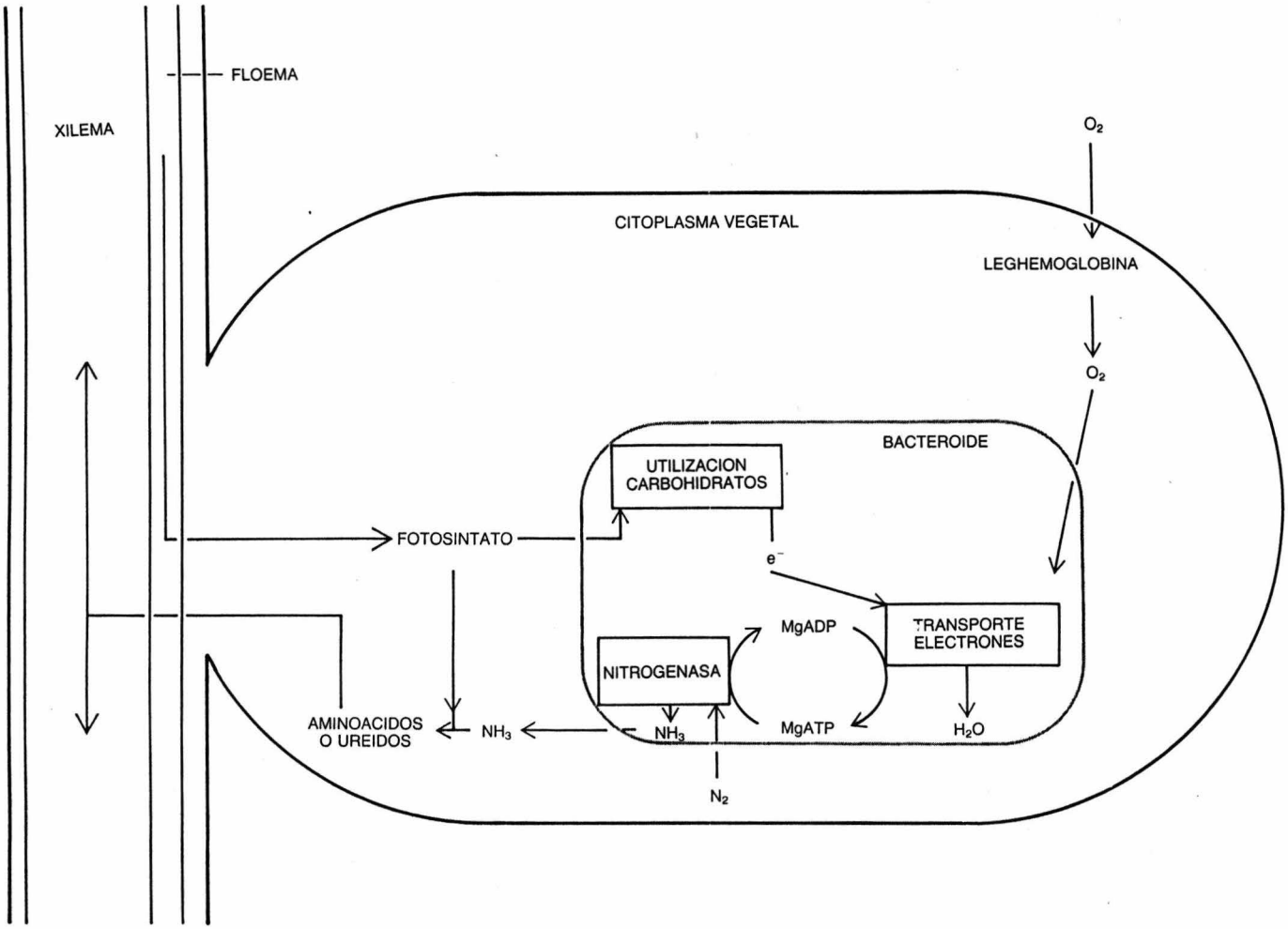
La obtención de mutantes defectivos en el establecimiento de la simbiosis, y su cartografía correspondiente, posibilitó localizar ese tipo de información en plásmidos. El grupo de investigación de J. Beringer y A. Johnston, del Instituto John Innes en Inglaterra, informó en 1980 que mutaciones localizadas en un plásmido transferible de *R. leguminosarum* dañaban la capacidad de nodular o de fijar nitrógeno dentro de los nódulos.



2. CICLO DEL NITROGENO. La fuente de nitrógeno que utilizan los distintos organismos varía; la serie de transformaciones que sufre el nitrógeno al ser asimilado constituye un ciclo. La entrada de este elemento a la biosfera depende de la conversión del nitrógeno atmosférico en amonio, proceso que recibe el nombre de fijación del nitrógeno. Sólo ciertas bacterias son capaces de usar el N_2 para desarrollarse.

En 1976, R. Dixon, de la Universidad inglesa de Sussex, aisló en un plásmido conjugativo la región del cromosoma de *Klebsiella pneumoniae* que determina la síntesis de histidina (*his*) y los genes que participan en la fijación de nitrógeno (*nif*); al transferir este plásmido a bacterias como *Escherichia coli* y *Salmonella typhimurium*, recibían éstas la capacidad de fijar nitrógeno.

Conocemos ya con bastante pormenor la región del cromosoma de *Klebsiella pneumoniae* que contiene los genes *nif*. Se ha identificado 17 genes, de muchos de los cuales se sabe su fun-



3. DE LA INTERACCION entre *Rhizobium* y la raíz de una leguminosa se forma un nódulo. En él la bacteria se convierte en bacteroide y fija nitrógeno. Por su lado, la planta aporta el fotosintato para la actividad de la enzima nitrogenasa, que asimilará el amonio y proporcionará alimento a aquélla.

ción. El trabajo experimental lo han llevado adelante los grupos de investigación de la Universidad de Sussex en Inglaterra y de la Universidad de Wisconsin en los Estados Unidos. En 1977, F. Cannon y F. Ausubel clonaron toda la región *nif* de *Klebasiella pneumoniae* en plásmidos, que se encuentran en muchas copias por célula. Los 17 genes se partieron en varios fragmentos; se aislaron cinco clones independientes que venían a cubrir la región *nif* entera; en un clon de éstos se identificaron los tres genes que codifican para la enzima nitrogenasa.

F. Ausubel publicaba en 1980 que el ADN del clon que lleva los genes estructurales de la nitrogenasa de *Klebsiella pneumoniae* hibridizaba con el ADN de distintas bacterias fijadoras del nitrógeno; dentro de éstas las había de diferente linaje evolutivo: cianobacterias y clostridias; demostrábase así que la nitrogenasa era una enzima que persistía a lo largo de la evolución biológica. Las secuencias del ADN de *Rhizobium* homólogas a los genes estructurales de la nitrogenasa de *K. pneumoniae* se encuentran codificadas en plásmidos, como se ha demostrado en varias especies: *R. leguminosarum, R. trifolii, R. phaseoli* y *R. meliloti*.

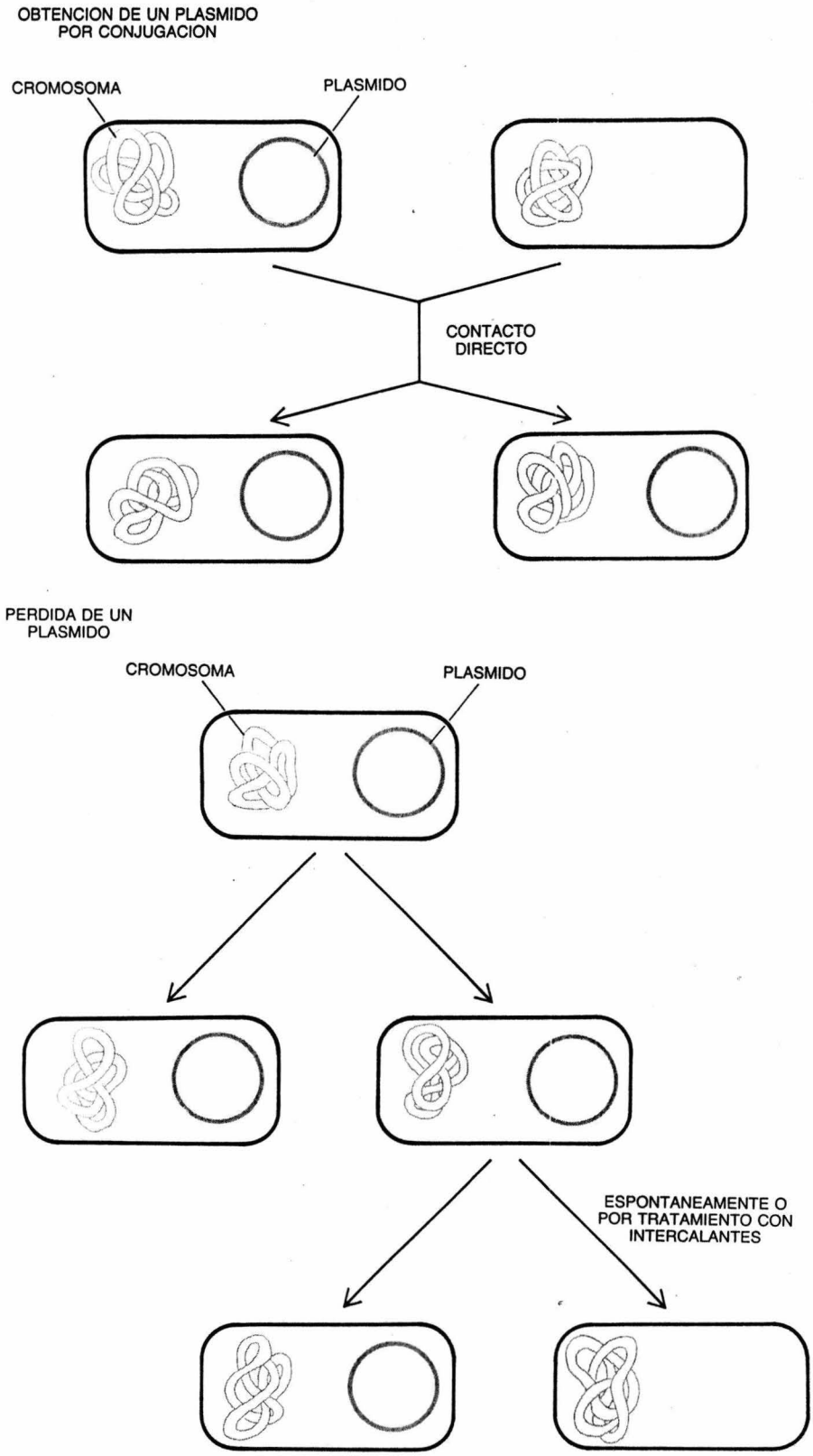
En 1982 se informó del trabajo realizado en el Centro de Investigación sobre Fijación de Nitrógeno de la Universidad Nacional Autónoma de México, según el cual se repetían las secuencias de ADN de *Rhizobium phaseoli* homólogas a los genes estructurales de la nitrogenasa de *K. pneumoniae*. Se sabe que en esta especie existen tres regiones con genes *nif*, todas codificadas en un mismo plásmido. El fenómeno de reiteraciones de genes *nif* se ha observado también en otras bacterias fijadoras de nitrógeno. No se conoce aún el significado biológico de la reiteración de estos genes.

En algunas cepas de *Rhizobium* se ha demostrado que el plásmido que lleva información para la nodulación es el mismo que tiene los genes estructurales de la nitrogenasa. A este tipo de plásmidos se les denomina *Sym* (simbióticos). Recientemente se han aislado genes que están involucrados en el proceso de nodulación de algunas especies de *Rhizobium*, todos ellos presentes en el plásmido *Sym*. Este trabajo lo realizaron A. Kondorosy y S. Long con *R. meliloti* y A. Downey con *R. leguminosarum*. La identificación de los productos de estos genes podría llevar al entendimiento del proceso de modulación en un nivel molecular.

Los plásmidos son moléculas informacionales que se encuentran en la inmensa mayoría de las bacterias gram negativas. Como se verá más adelante, dadas sus características estructurales los plásmidos tienen características distintas de cualquier otro tipo de moléculas de ADN.

El primer plásmido descrito en bacterias fue el factor *F*, descubierto en los primeros años de la década de los 50 por Lederberg y Hayes al estudiar la conjugación bacteriana. Observaron que, para llevar a cabo esta transferencia, era necesario que la bacteria donadora tuviera el factor *F*; se requería, además, el contacto directo entre dos bacterias. Si el factor *F* se encontraba integrado en el cromosoma bacteriano, promovía la transferencia de genes cromosómicos, si no se transfería sólo el

mismo. En 1956, François Jacob y Wollman demostraron que el factor *F* era una molécula de ADN que se autorreplicaba y que se agregaba a la información genética bacteriana; una bacteria podía obtener, así, un plásmido por conjugación, y debía perderlo para regresar a su estado anterior. La capacidad de promover la conjugación bacteriana constituye una peculiaridad exclusiva de los plásmidos; existen, empero, un grupo de estos elementos, los plásmidos no conjugativos, que son incapaces de transferirse.



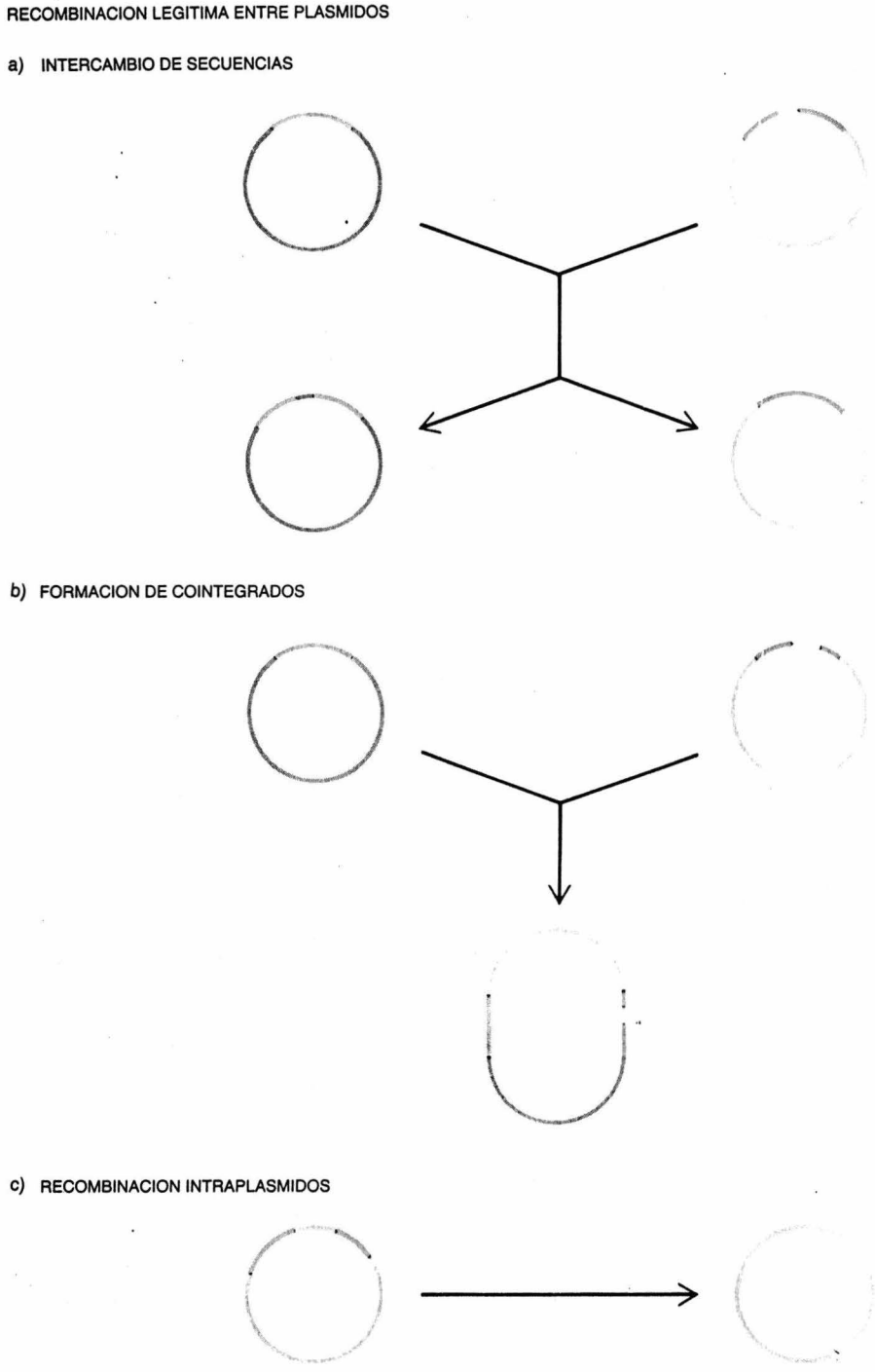
4. GANANCIA Y PERDIDA DE PLASMIDOS. Son éstos moléculas de ADN capaces de autotransferirse entre bacterias. La información que encierran se suma así a la que contiene el cromosoma bacteriano hospedante (*arriba*). Una bacteria puede perder un plásmido espontáneamente o por el uso de agentes químicos como intercalantes o agentes físicos como el calor (*abajo*), regresando a su estado original.

En 1959 se encontró el segundo gran grupo de plásmidos, los plásmidos *R*, que determinan resistencia a antibióticos. Identificados en Japón durante una epidemia de disentería, en la que se aislaban de los enfermos cepas con múltiples resistencias, entre ellas a antibióticos que no habían sido usados para el tratamiento. Este fenómeno les hizo pensar que tales resistencias se habían coseleccionado junto con las adquiridas contra los antibióticos administrados a los enfermos; la explicación de tal coselección había que buscarla en que todos se codificaran por la misma molécula de ADN. Posteriormente, se determinó que todas las resistencias se transferían juntas en cepas de *Shigella disenteriae*; de ésta pasaban a otro tipo de bacterias, como *Escherichia coli*.

Con los años fueron descubriéndose muchos tipos de plásmidos. Presentan, además de su capacidad de transferencia y resistencia a antibióticos, múltiples virtualidades: codifican la producción de bacteriocinas, determinan la patogenecidad de las bacterias (así, en ciertos serotipos patógenos de *E. coli* o en *Agrobacterium tumefaciens*, que produce tumores en un gran número de plantas), etcétera. En ciertos medios, contener un plásmido representa una ventaja selectiva para la bacteria; por ejemplo, cuando tiene un plásmido *R* y está en presencia de un antibiótico contra el que le confiere resistencia.

En la célula bacteriana pueden coexistir varios plásmidos siempre que no tengan un mecanismo de replicación igual que les induzca a la mutua competencia. Este fenómeno de incompatibilidad constituye uno de los criterios utilizados para la clasificación de plásmidos. Son éstos moléculas muy plásticas. Atraviesan reorganizaciones estructurales que crean configuraciones con características nuevas. Participan frecuentemente en procesos de recombinación.

Existen dos grandes mecanismos de recombinación de ADN. Exige el primero que las secuencias a intercambiar presenten una estrecha homología (recombinación legítima); el segundo mecanismo sucede en ausencia de homología (recombinación ilegítima). La recombinación legítima puede seguir varios caminos, pero todos dependen de la función del producto de un gen denominado *recA*; del que no necesita la ilegítima para desarrollarse. Aunque no se conoce bien el mecanismo por el que se lleva a cabo esta función, se sabe que el producto del gen *recA* participa en todos los procesos celulares donde in-



5. RECOMBINACION LEGITIMA ENTRE PLASMIDOS. Si se encuentran dentro de una bacteria secuencias de ADN homólogas, participan en procesos de recombinación. En algunos casos, los plásmidos que coexisten en una célula comparten secuencias; ello les permite intercambiar información o formar cointegrados. Las secuencias homólogas del mismo plásmido pueden perderse por recombinación.

terviene la recombinación legítima; en su ausencia, es muy bajo el rendimiento de la recombinación entre secuencias homólogas presentes en el cromosoma bacteriano.

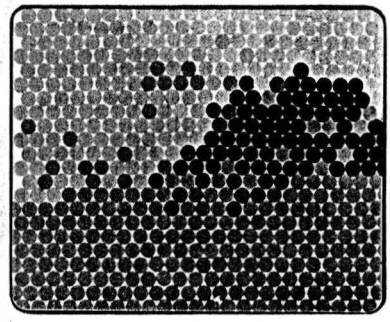
Cuando coexisten en una célula dos plásmidos de secuencia homóloga se recombinan e intercambian información o bien se cointegran para formar una sola molécula, que es la suma de ambos plásmidos. La recombinación entre secuencias homólogas se produce a veces también dentro de la misma molécula. Propio de algunos plásmidos es que pueden participar en fenómenos de recombinación legítima en ausencia del producto del gen *recA*.

La recombinación ilegítima o transposición es la que presentan las llamadas secuencias de inserción o los transposones. Durante la transposición, las secuencias de ADN cambian de lugar en el genoma y se insertan en una región que no guarda homología con ella; si esta integración sucede dentro de un gen se produce una mutación, pues se rompe la continuidad de la información codificada. Todos los transposones y

Carleton S. Coon

ADAPTACIONES RACIALES

Un estudio de los orígenes, naturaleza y significado de las variaciones raciales en los humanos



Un volumen de 224 páginas, con numerosas ilustraciones en negro y color.
Colección Labor Universitaria, *Manuales*

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Carletón S. Coon era una de las personas que más sabían sobre la variabilidad humana. Precisamente cuando se estaba preparando la edición de esta obra, en el verano de 1981, Coon falleció.

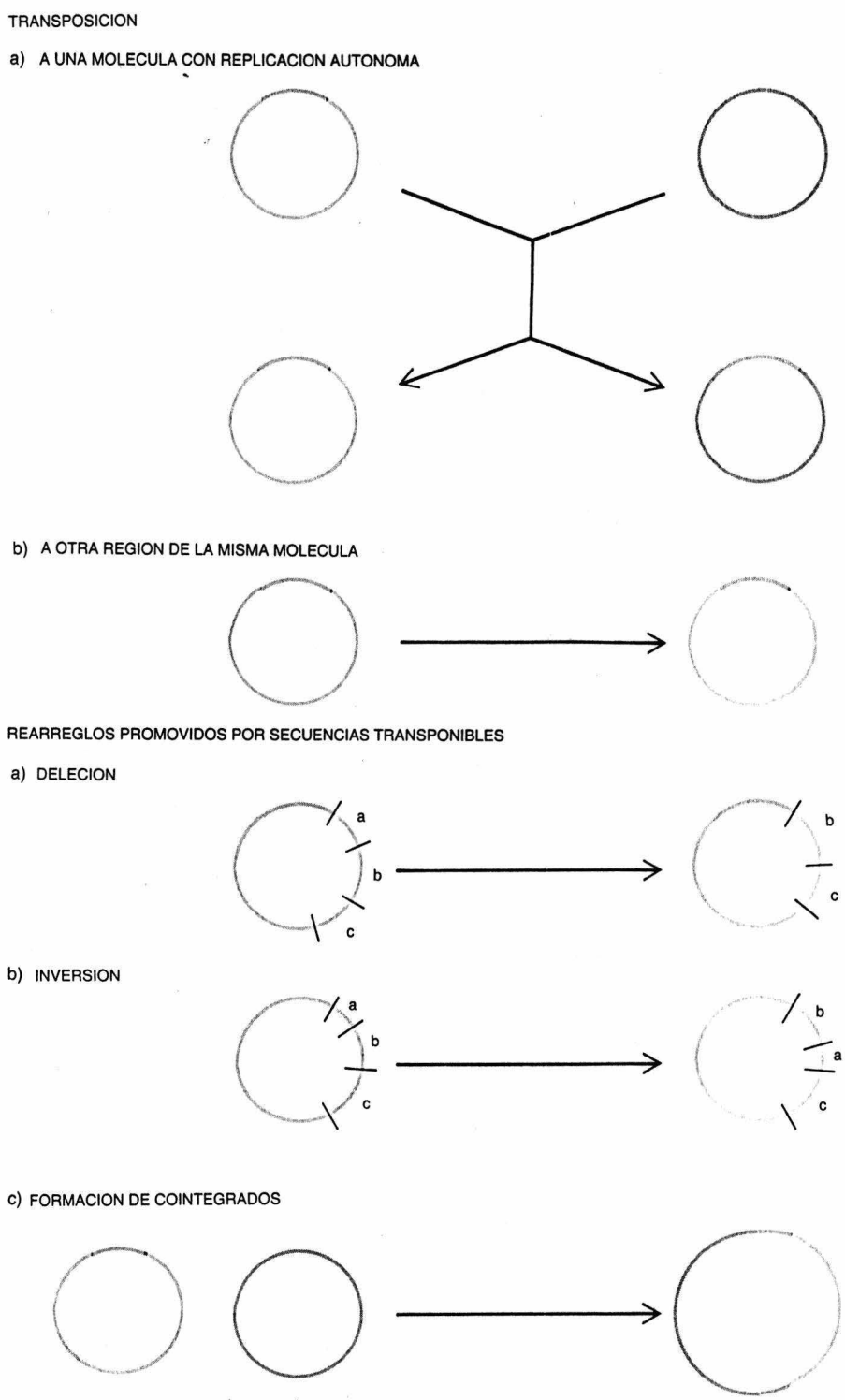
El libro es una interesante colección de meditaciones y juicios sobre la naturaleza y significado de las variaciones raciales. Se trata de un libro de ideas y no de una compilación de fechas y referencias de tipo enciclopédico; ésta es la característica de los anteriores escritos de Coon: *The Races of Europe* (1939), *Las razas humanas actuales* (Guadarrama, 1969) y *The Living Races of Man* (1965).

Las variaciones físicas, fisiológicas y genéticas en los seres humanos suelen crear controversias, porque a menudo se confunden con ideas sociales y políticas. Pero cualquiera que sea nuestra idea sobre la raza, la diversidad humana existe, y sólo una pequeña parte de ésta ha sido relacionada con los factores ambientales.

Los antropólogos saben muy bien que la luz intensa favorece la piel obscura y que la malaria endémica puede ser causa de selección por la mayor frecuencia de hemoglobinas anormales en los glóbulos rojos. Otros factores, como los mecanismos por los cuales la talla de los cuerpos puede estar relacionada con la función de la hipófisis y con el agua, el calcio y el metabolismo del fósforo, no están muy bien determinados. Justamente, uno de los más interesantes y valiosos aspectos de este libro son las sugerencias que hace Coon sobre estos temas.

Tanto desde el punto de vista de una introducción al estudio de la variabilidad humana, como por tratarse de la última obra de un eminente antropólogo, este libro merece, sin duda, la lectura minuciosa de todos los que se interesan por la antropología.

LABOR



6. REARREGLOS PROMOVIDOS POR TRANSPOSICION. Llamados también reordenaciones cromosómicas. Durante la transposición, una secuencia de ADN cambia de posición en el genoma y se inserta en una secuencia con la que no tiene homología. La presencia de transposones aumenta la frecuencia de rearreglos estructurales (deleciones, inversiones o formación de cointegrados) del ADN donde se halla.

las secuencias de inserción están flanqueados por secuencias invertidas repetidas; en algunos casos se sabe que determinan los productos que intervienen en su transposición.

Las secuencias que son capaces de transponer promueven reordenaciones estructurales de las moléculas en las que están insertas; a saber: deleciones, inversiones y uniones. Por cuya razón las moléculas de ADN que contienen secuencias de inserción o transposones son menos estables que las que carecen de este tipo de secuencias. Los plásmidos son moléculas de ADN donde suelen darse secuencias transponibles; de hecho, la gran mayoría de transposones descritos forman parte estructural de algún plásmido, razón de más para la plasticidad de este tipo de moléculas.

En varias especies de *Rhizobium*, parte de la información que determina el establecimiento de la simbiosis con la leguminosa está codificada en plásmidos, lo que comporta diversas implicaciones para su estabilidad, distribución entre diferentes bacterias y su capacidad de asociarse con otro tipo de información. Se han encontrado reordenaciones estructurales donde intervienen los plásmidos *Sym* de *Rhizobium*, pero no se ha determinado si son producto de recombinación legítima o no; tampoco se sabe si estas moléculas encierran secuencias transponibles.

El grupo de B. Rolfe, de la Universidad Nacional de Australia, expuso que un plásmido *Sym* de *R. leguminosarum* sufría deleciones al encontrarse dentro de una cepa de *R. trifolii*. Tales reordenaciones ocurrían con más frecuencia cuando la bacteria estaba en contacto con la planta hospedadora que cuando crecía en un medio de cultivo. El grupo de Rafael Palacios, del Centro de Investigación sobre Fijación de Nitrógeno de la Universidad Nacional Autónoma de México, encontró que al someter una cepa de *R. phaseoli* a un tratamiento para eliminar plásmidos era muy frecuente encontrar un cointegrado entre el plásmido *Sym* y otro de menos peso molecular. Por mi parte, trabajando en el mismo Centro de Investigación, hallé que el plásmido *Sym* de una cepa de *R. phaseoli* que pierde la capacidad de nodular fríjol a muy alta frecuencia presenta rearreglos estructurales y que la bacteria que contiene el plásmido rearreglado pierde la capacidad de nodular fríjol.

La participación de los plásmidos *Sym* en procesos de recombinación en condiciones naturales nos lo sugiere el hecho de que existan dos de estas moléculas que establecen la simbiosis con una especie de leguminosa, sin dejar de portar su información peculiar y distinta. Me refiero a los plásmidos *Sym* de dos cepas de *R. leguminosarum*, estudiados por J. Beringer, A. Johnston y N. Brewin, en el Instituto Johns Innes. Uno de ellos es transferible y determina la producción de bacteriocinas; el otro, que no es conjugativo, contiene los genes de la hidrogenasa de captura.

Si la información para la simbiosis puede estar asociada con información para otros procesos, cabe que, por recombinación, este tipo de secuencias estén repartidas en dos o más plásmidos independientes. Tal sucede en una cepa de *R. phaseoli*. En el Centro de Investigación sobre Fijación de Nitrógeno de la Universidad Nacional Autónoma de México, J. Leemans y M.

Mejías aislaron derivados de esta cepa que tienen defectos en el establecimiento de la simbiosis con el fríjol por la pérdida de un plásmido y que conservan otro que determina los genes estructurales de la nitrogenasa.

La información para el establecimiento de la simbiosis entre *Rhizobium* y la leguminosa está codificada, por lo menos en parte, en plásmidos. Estos plásmidos *Sym*, en condiciones de laboratorio, son moléculas muy plásticas. Ahora bien, aunque existen fenómenos en la naturaleza en los que podría tener importancia la codificación en plásmidos de la información simbiótica de *Rhizobium*, esto no se ha explorado directamente.

Entre estos fenómenos citemos la ineficacia de algunas prácticas de inoculación de leguminosas de interés comercial. A pesar de que se utilizan bacterias que en condiciones de invernadero son muy buenos simbiontes, en ocasiones se observa que en el campo este *Rhizobium* forma una fracción pequeña de los nódulos o pierde su capacidad para fijar nitrógeno con el paso del tiempo. Este problema puede deberse a que el plásmido *Sym* de la bacteria usada como inoculante sea inestable en las condiciones de campo, a que no se transfiera a otras bacterias presentes en el suelo o a que en estas condiciones participe en sucesos de recombinación que generen plásmidos con defectos en la información simbiótica.

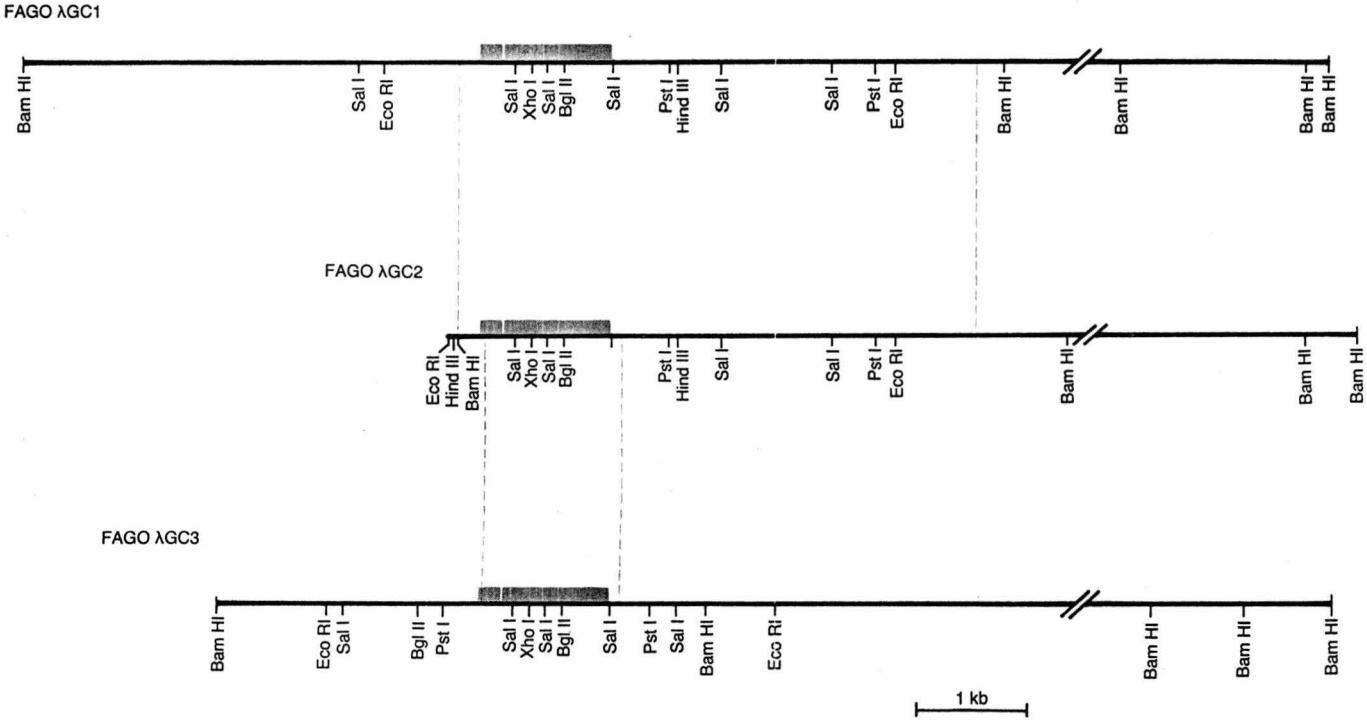
El número de los *Rhizobia* específicos para una leguminosa aumenta cuando ésta se cultiva. La información simbiótica se podría transferir por conjugación entre bacterias, de tal manera que la multiplicación de los simbiontes no sólo dependería de la capacidad de las bacterias para replicarse cuando se encuentra presente la planta hospedante, sino que se reclutarían nuevos *Rhizobia* entre las bacterias nativas del suelo que heredarán el plásmido *Sym*.

Otro fenómeno es el de la aparente dispersión evolutiva de los *Rhizobia* pertenecientes a una especie, como es el caso de *R. phaseoli*. Esta observación podría deberse a que el plásmido *Sym* de esa especie pueda funcionar en fondos genéticos muy variados, de suerte que bacterias alejadas evolutivamente permitan la expresión del plásmido y puedan formar nódulos en fríjol.

Actualmente se puede estudiar directamente la participación de los plásmidos *Sym* en los acontecimientos mencionados, ya que se cuentan con marcadores genéticos tanto en el cromosoma bacteriano como en diversos sitios del plásmido *Sym*. Por otra parte se puede hacer un análisis evolutivo de distintos plásmidos *Sym* para determinar la participación de eventos de recombinación en su generación o mantenimiento; este enfoque experimental lo está abordando el grupo de investigación de Rafael Palacios en la Universidad Nacional Autónoma de México.

La información genética de *Rhizobium* que determina el establecimiento de la simbiosis con las leguminosas está codificada en plásmidos de alto peso molecular; por ello presenta características peculiares para su distribución y mantenimiento; se puede transferir entre bacterias por conjugación y puede segregar del cromosoma bacteriano y participar en eventos de recombinación que afecten su estructura. Estas características propias de los elementos extracromosómicos repercuten en el manejo de *Rhizobium* como inoculante en plantas de interés comercial.

Al contemplar en *Rhizobium* la interacción entre dos genomas con propiedades diferentes cabe plantear enfoques experimentales que descifren la dinámica de población de estas bacterias en el suelo y permitan la selección de cepas idóneas para su empleo como inoculantes. Por otra parte, se puede aplicar a *Rhizobium* las técnicas de manipulación genética de bacterias, tales como la recombinación *in vitro* de ADN, al objeto de construir plásmidos *Sym* híbridos y seleccionar el mínimo de información que le permitiera a una bacteria del suelo fijar nitrógeno en asociación con una leguminosa sin ser desplazada por los *Rhizobia* presentes en la tierra.



7. MAPA DE RESTRICCION de las tres regiones del plásmido *Sym* de la cepa CFN42 de *R. phaseoli* que contienen los genes estructurales de la nitrogenasa. Se presentan los mapas de los insertos en los fagos λGC1 (a), λGC2 (b) y λGC3 (c). Las barras corresponden a las zonas secuenciadas en las que se resalta (*en gris*) la zona que determina la nitrogenasa reductasa (*nif H*); la homología estructural entre las regiones se muestra entre líneas punteadas. *E* corresponde a Eco RI, *H*, a Hind III; *G*, a Bgl II; *X*, a Xho I; *B*, a Bam HI; *P*, a Pst I; *S*, a Sal I. (Mapa proporcionado por Carmen Quinto, de la UNAM.)

TITLTE: Instability of *Rhizobium phaseoli* symbiotic properties

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SUMMARY

Twenty two *Rhizobium phaseoli* isolates were analized for the stability of their symbiotic properties after a treatment to cure plasmid; four of these strains loose their ability to nodulate beans at a high frequency (≥32.5%); three of the symbiotically unstable *R. phaseoli* strains were isolated from the same agricultural region. The prevalence of a particular, simbiotically unstable strain was discarded by the analisis of the plasmid eletrophoretic pattern of the unstable *R. phaseoli* isolates. The restriction pattern of the nitrogenase reductase genes (*nifH*) was analized, the symbiotically unstable *R. phaseoli* strains have the same restriction pattern, while five stable *R. phaseoli* isolates analized have a variable restriction pattern of these genes. The non nodulating (Nod^-) derivatives analized loose *nifH* genes, but this loss of DNA could not be correlated with a change in the electrophoretic pattern of their plasmid. This type of Sym plasmid deletion has been described for one *R. phaseoli* strain (Soberón-Chávez *et al*, 1986).

INTRODUCTION

Bean inoculation with selected *Rhizobium phaseoli* strains has not been a successfull agronomic practice (Graham, 1981), the reason for the failure of these bacteria to provide enough nitrogen to sustain plant growth, is not clear (Martinez and Palacios, 1984).

Rhizobium phaseoli symbiotic information is encoded in a plasmid (Sym plasmid), (Hombrecker *et al*, 1981). This Sym plasmid has a peculiar genetic structure; it has been shown that most of the bacteria isolated from nitrogen fixing bean nodules contain reiterated nitrogenase structural genes (Martínez *et al*, 1985), and many other DNA sequences also present in the Sym plasmid are also reiterated (Flores *et al*, 1985).

In some *R. phaseoli* strains the Sym plasmid participates in different genetic rearrangements that modify the bacteria's symbiotic phenotype (Soberón-Chávez *et al*, 1986). One of these rearrangements is the deletion of the symbiotic plasmid, this plasmid looses ≈100kb of DNA including the three copies of the nitrogenase reductase gene (*nifH*), but the apparent molecular wheight of this plasmid remains unaltered. The bacteria which habours the deleted symbiotic plasmid is unable to nodulate beans.

The genetic plasticity of the Sym plasmid could cause

that the nodule forming ability of *Rhizobium phaseoli* isolates is lost at a high frequency and this may be related to the failure of these bacteria to increase bean yield when used as inoculant in the field.

We found that three out of six *R. phaseoli* isolated from Celaya, Guanajuato, a Mexican region where beans are currently cultivated, loose the ability nodulate beans of a high frequency (see RESULTS and DISCUSSION), so we decide to look for the presence of symbiotically unstable *R. phaseoli* isolates in other Mexican regions where beans are currently cultivated. We were unable to show a correlation between the presence of symbiotically unstable *R. phaseoli* strains and the failure of this bacteria to increase plant yield when used as inoculants.

We analized the plasmid electrophoretic profile and *nifH* restriction pattern of the unstable *R. phaseoli* isolates and showed that a similar molecular mechanism to that described for the instability of the symbiotic phenotype of *R. phaseoli* CFN23 strain (Soberón-Chávez *et al*, 1986) is involved in the loss of the symbiotic phenotype of all the unstable *R. phaseoli* isolates.

METHODS

Isolation of *Rhizobium phaseoli* strains. Bacteria were isolated from bean nodules colected in Celaya, Guanajuato; Zacatepec, Morelos or Oaxaca, Oaxaca as shown in Table 1; each nodule was surface sterilized, crushed and streaked in PY media (Noel *et al*, 1984), a single colony was isolated from each nodule and tested for bean nodulation in laboratory conditions (Martínez *et al*, 1985). The frequency of loss of the nodule forming ability of each isolate was quantitated as described below. The strains which were further characterized are shown in Table 2.

Determination of Symbiotic Instability. Each of the *Rhizobium phaseoli* strains was streaked in YM media (Vicent, 1970) and incubated at 37°C for five days. This treatment has been shown to enhance the frequency of loss of the symbiotic properties of a *Rhizobium phaseoli* strain (Soberón-Chávez *et al*, 1986), and was original described to cure *Rhizobium trifolii* plasmids (Zurkowski, 1982). Eight single colonies were purified from each strain that was incubated at 37°C, and their ability to nodulate beans was tested; to avoid false negative results, all the bacteria not able to form nodules (Nod^-) in the first screening were inoculated into beans for a second test. A strain was consider to be symbiotically unstable if at least three of

the eight colonies tested were Nod$^-$.

Isolation and Manipulation of DNA. Conditions for isolation of DNA, and Southern blot hybridization were those reported by Quinto *et al*, 1982. *R. phaseoli* probe was described previously (Martínez *et al*, 1985). Plasmids were visualized by the proceedure described by Eckhardt, 1978.

RESULTS and DISCUSSION

Simbiotically unstable *R. phaseoli* isolates are only common in one agricultural field. The incubation of *Rhizobia* at 37°C for several days increases the loss of the symbiotic plasmid of *R. trifolii* strains (Zurkowzki, 1982), in order to obtain *Rhizobium phaseoli* derivatives cured of their symbiotic plasmid, we heat treated six *R. phaseoli* strains isolated in Celaya, Guanajuato, México (Table 1); we found that three of these strains (CFN1, CFN5 and CFN23, Table 2) loose their ability to nodulate beans at a very high frequency; more than 30% of the colonies tested after hear treatment were unable to nodulate beans. (data not shown)

In order to determine if the high frequency of loss of the symbiotic phenotype of *Rhizobium phaseoli* isolates was related with the ineficiency of these bacteria when used as inoculants in the field, we studied the frequency of unstable *R. phaseoli* isolated from other two Mexican agricultural regions where beans are currently cultivated (Zacatepec, Morelos and Oaxaca, Oaxaca, Table 1), the three agricultural regions chosen were more than 500kb appart from each other. We found that only one *Rhizobium phaseoli* strains was symbiotically unstable among the sixteen strains isolated in Zacatepec, Morelos and Oaxaca, Oaxaca (Table 1), this result shows that symbiotically unstable

R. phaseoli strains are not only found in Celaya, Guanajuato, but that their frequency is lower in other agricultural regions, so the instability of the symbiotic phenotype does not seem to be related to the lack of increase of bean yield when *R.phaseoli* is inoculated into the soil.

A similar molecular mechanism of the loss of the nodulation ability of *Rhizobium phaseoli* is present in different strains. One of the symbiotically unstable *R. phaseoli* strains (CFN23, Table 2) has been characterized at a molecular level (Soberón-Chávez, 1986). In order to determine if the other three symbiotically unstable *R. phaseoli* strains (CFN1, CFN5 and CFN 990, Table 2) loose their ability to nodulate beans by a similar mechanism to that of the deletion affecting the symbiotic plasmid of CFN23 strain, the plasmid electrophoretic pattern and the restriction patter of the nitrogenase reductase genes (*nifH*) of these strains were analized and compared with those of their non nodulating (Nod^-) derivatives. We found (Fig. 1) that the plasmid electrophoretic pattern of the Nod^- derivatives was identical to that the original strains, nevertheless a deletion of the symbiotic plasmid of all the Nod^- derivatives was apparent since the four strains loose the three *nifH* copies; these results show that the high frequency of loss of the symbiotic phenotype of *R. phaseoli* strains is due to a similar molecular mechanisms to that reported for CFN23 strain.

The prevalence of a particular symbiotically unstable *R. phaseoli* strains was discarded since the plasmid eletrophoretic pattern of the strains was not the same, (Fig. 1); only CFN23 strain and CFN5 strain have the same plasmid electrophoretic profile and could be a prevalent strain in Celaya, Guanajuato.

The symbiotically unstable *R. phaseoli* isolates contain a similar symbiotic plasmid. The *nifH* restriction pattern of the four symbiotically unstable strain is identical (Fig. 1), and is not the most common restriction pattern among the *R. phaseoli* isolates (Martínez *et al*, 1985). We hybridize the plasmid electrophoretic pattern of the symbiotically unstable strains with *nifH* probe and found that the four contain a 400kb hybridizing plasmid (data not show). The identity of the *nifH* restriction pattern and that of the symbiotic plasmid molecular weight among the symbiotically unstable strains studied, suggest that these strains contain a similar symbiotic plasmid, eventhough not all have the same geographical origin.

To rule out the possibility that the *nifH* restriction pattern found in the symbiotically unstable strains was the most common pattern among our *R. phaseoli* isolates, we analized five *R. phaseoli* strains that do not loose their symbiotic phenotype at a high frequency, we found that only one of these stable strains (CFN410, Table 2), share the *nifH* restriction

pattern of the symbiotically unstable *R. phaseoli* isolates (Fig. 2).

We were not able to show a correlation between the existance of symbiotically unstable *R. phaseoli* strains and the inability of these bacteria to increase plant yield when used as inoculant in the field, but we are developing an experimental model to evaluate the importance of genetic rearrangements in the stablishment of the symbiosis between *Rhizobium phaseoli* and common beans, studying these bacteria directly in soil, their natural habitat.

ACKNOWLEDGMENTS

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Table 1. Frequency of symbiotically unstable *R. phaseoli* strains in different Mexican agricultural regions

Region	Number of strains analized	Number of unstable strains
Celaya, Guanajuato	6	3
Zacatepec, Morelos	12	1
Oaxaca, Oaxaca	4	0
T o t a l	22	4

Table 2. *Rhizobium phaseoli* strains further analized.

Strain	Relevant characteristic	Please of Isolation
CFN1	looses its ability to nodulate frequently	Celaya, Guanajuato
CFN5	looses its ability to nodulate frequently	Celaya, Guanajuato
CFN23	looses its ability to nodulate frequently	Celaya, Guanajuato
CFN990	looses its ability to nodulate frequently	Zacatepec, Morelos
CFN42	its ability to nodulate is stably mantained	Celaya, Guanajuato
CFN410	its ability to nodulate is stable mantained	Zacatepec, Morelos
CFN840	its ability to nodulate is stable mantained	Zacatepec, Morelos
CFN935	its ability to nodulate is stable mantained	Oaxaca, Oaxaca
CFN942	its ability to nodulate is stable mantained	Oaxaca, Oaxaca

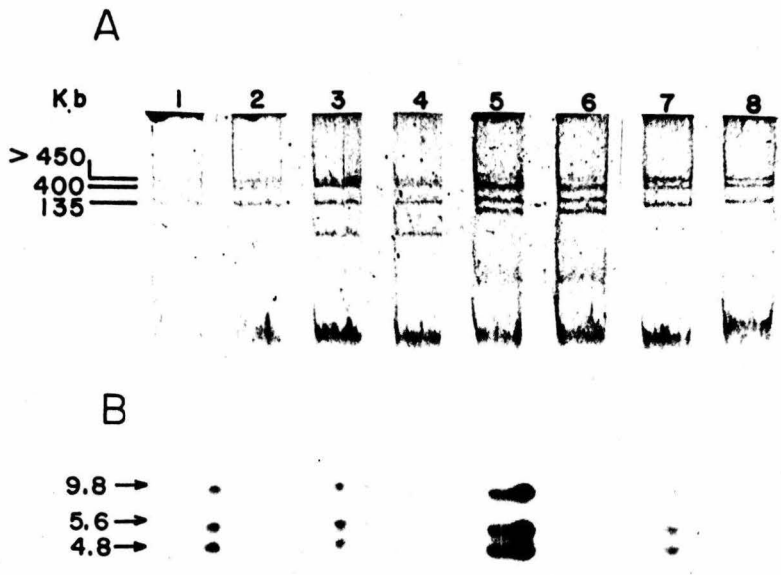

Figure 1

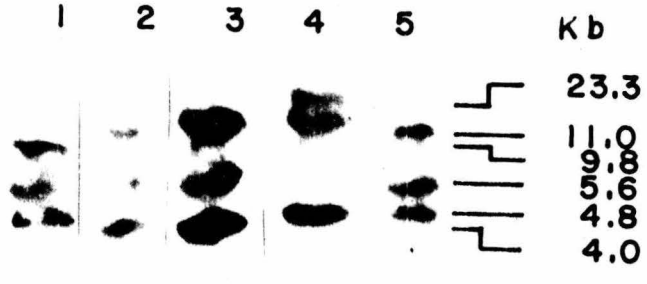

Figure 2

FIGURE LEGENDS

Figure 1. (A) Plasmid eletrophoretic pattern on 0.7% agarose gels stained with ethidium bromide and (B) *nifH* hybridization of total cellular DNA diggested with BamHI endonuclease of symbiotically unstable *R. phaseoli* isolates and their non nodulating derivatives (Nod^-). Lanes correspond to the following strains: 1) CFN23 2) CFN23 Nod^- 3) CFN1 4) CFN1 Nod^- 5) CFN990 6) CFN990 Nod^- 7) CFN5 8) CFN5 Nod Numbers correspond to molecular weight of DNA sequences in kilobases (kb).

Figure 2. *nifH* hybridization of total cellular DNA digested with BamHI endonuclease of *R. phaseoli* strains which stably m antain their symbiotic phenotype. Lanes correspond to the following strains 1) CFN410 2) CFN42 3) CFN840 4) CFN932 5) CFN942. Numbers correspond to molecular weights of the hybridizing DNA sequences in kilobases (kb).

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Genetic Rearrangements of a *Rhizobium phaseoli* Symbiotic Plasmid

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Different structural changes of the Sym plasmid were found in a *Rhizobium phaseoli* strain that loses its symbiotic phenotype at a high frequency. These rearrangements affected both *nif* genes and Tn*5 mob* insertions in the plasmid, and in some cases they modified the expression of the bacterium's nodulation ability. One of the rearrangements was more frequent in heat-treated cells, but was also found under standard culture conditions; other structural changes appeared to be related to the conjugal transfer of the plasmid.

The genus *Rhizobium* comprises the gram-negative bacteria that form nodules on legumes. In this association, the bacteria fix atmospheric nitrogen that is then assimilated by the plant.

The genetic information controlling symbiotic activity in the fast-growing rhizobia is encoded in plasmids (10, 11, 13, 19). A symbiotic (Sym) plasmid has been defined as one that determines the plant species specificity for nodulation and contains the nitrogenase enzyme structural genes (*nif* genes) (12).

Plasmids participate very frequently in recombination events (14, 20). It has been proposed (27) that this plasticity enables the bacteria that harbor plasmids to adapt to different environmental changes and permits the rapid spread of newly created functions among very diverse bacteria. There is one report (24) of a change in the structure of a plasmid that resulted in modified metabolic activity in the recipient bacteria. This strongly suggests that plasmid plasticity is important in the generation of new functions in bacteria.

In *Rhizobium phaseoli*, the nitrogen fixation gene sequences are reiterated (25). In strain CFN42, there are three regions of the Sym plasmid that contain nitrogenase structural genes (*nif* regions) (22). These three regions contain the nitrogenase reductase gene (*nifH*); the nucleotide sequence of the three copies is identical (26). In addition, two of the regions contain also *nifD* and *nifK* genes. The identity of the *nifH* genes suggests that a recombination event could be involved in the generation or maintenance of their reiteration. Reiterated sequences are not common in bacteria, but they have been found in some strains of *R. phaseoli*, *Rhizobium trifolii*, and *Rhizobium japonicum* (2) and also *Streptomyces* sp. (21), *Halobacterium halobium* (29), and *Pseudomonas syringae* p.v. "*phaseolicola*" (32). The presence of these reiterations may be related to the instability and genetic rearrangements of these organisms (1, 6, 23).

We have found that symbiotically unstable isolates are very common among the *R. phaseoli* strains isolated in different regions of Mexico, and all have reiterated *nifH* genes (L. Castrejón and G. Soberón, manuscript in preparation). We suppose that the symbiotic instability of these *R. phaseoli* strains is due to genetic rearrangements caused by the presence of reiterated sequences. We report here that the loss of the symbiotic phenotype of an unstable *R. phaseoli* strain is due to changes in the structure of its Sym plasmid, and that this Sym plasmid can be involved in different rearrangements which modify the information it carries.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are shown in Table 1. The important characteristics of their isolation are described below.

Growth conditions. PY medium (18) was used to grow *R. phaseoli* strains, unless otherwise stated. *Agrobacterium tumefaciens* and *Escherichia coli* strains were grown in LB medium (17). Both *R. phaseoli* and *A. tumefaciens* strains were grown at 30°C, and *E. coli* strains were grown at 37°C. The antibiotic concentrations used were as follows: cloramphenicol, 30 μg/ml; erythromycin, 50 μg/ml; gentamicin, 40 μg/ml; kanamycin, 60 μg/ml; rifampin, 30 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml.

Construction of pLS151. Plasmid pLS151 is a intermediary vector derived from plasmid pSUP205 (31) carrying Sp^r. pLS151 was constructed by inserting a *Bam*HI fragment conferring resistance to spectinomycin from plasmid R702 (15) into the *Bgl*II site of the *nifH* coding frame from *R. phaseoli* in plasmid pEM15 (E. Morett, manuscript in preparation); pEM15 carries a 4.9-kilobase (kb) *Eco*RI fragment comprising most of *R. phaseoli* CFN42 *nifH* region a (25, 26) subcloned in the *Eco*RI site of pSUP205. Quinto et al. (26) described the use of intermediary vectors similar to pLS151 to obtain interrupted *nifH* genes.

Construction of strain CFN2414. Strain CFN2414 was constructed by mobilization of plasmid pLS151 in a triparental mating with strain CFN2314 (Table 1) by using plasmid pRK2013 (8) as a helper plasmid; kanamycin- and spectinomycin-resistant transconjugants were isolated and scored for tetracycline sensitivity. Since the origin of replication of plasmid pSUP205 is not functional in *R. phaseoli*, the kanamycin-, spectinomycin-, and tetracycline-resistant transconjugants have the pLS151 plasmid cointegrated by a single recombination event with one of the *nif* regions in the CFN2314 Sym plasmid, whereas the transconjugants that are tetracycline susceptible have one of the wild-type *nif* regions in the CFN2314 Sym plasmid substituted by the mutated fragment in pLS151 in a double recombination event.

One such tetracycline-susceptible transconjugant was further analyzed in Southern blot hybridization experiments of total DNA digested with *Bam*HI versus an *nifH* specific probe and found to have the spectinomycin resistance DNA

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TABLE 1. Bacterial strains and plasmids

Strains or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
R. phaseoli CFN23	Nod$^+$ *nifH*$^+$	Field isolate from Mexico
R. phaseoli CFN2370[b]	Nod$^-$ Δ*nifH* Str^r	This work
R. phaseoli CFN2315[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r	This work
R. phaseoli CFN2350[b]	Nod$^-$ Δ*nifH* Rif^r Km^s	This work
R. phaseoli CFN2314[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r	This work
R. phaseoli CFN2414[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r Sp^r	This work
R. phaseoli CFN2340[b]	Nod$^-$Δ*nifH* Rif^r Km^r Sp^r	This work
A. tumefaciens GMI9023	Vir$^-$ (without plasmids) Str^r Rif^r	(28)
A. tumefaciens CFN2302[c]	Nod$^+$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens CFN2303[c]	Nod$^-$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens CFN2304[c]	Nod$^+$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens C58C1	Vir$^-$ Ery^r Cam^r	(5)
E. coli HB101	*recA hsdR hsdM*	(3)
Plasmids		
pLS151[d]	Vector for *nifH* sequences; carries Sp^r	This work
pRK2013	Tra$^+$ Km^r (not transposable); unable to replicate in rhizobia	(8)
pSUP5011	pBR325::Tn*5 mob* Km^r Ap^r Cm^r	(30)
pJB3J1	Tra$^+$ Tc^r Gm^r Km^s	(4)

[a] Abbreviations used: Nod, ability to nodulate beans; Vir, tumor-forming ability; Tra, conjugal transfer ability; *mob*, gene encoding the ability to be mobilized by some transferable plasmids; *nifH*, nitrogenase reductase gene. Resistances to rifampin (Rif^r), kanamycin (Km^r), erythromycin (Ery^r), cloramphenicol (Cam^r), tetracycline (Tc^r), gentamicin (Gm^r), and spectinomycin (Sp^r) and susceptibility to kanamycin (Km^s) and spectinomycin (Sp^s) are indicated. *recA* mutants are unable to recombine; *hrdR* and *hsdM* mutants are unable to modify or restrict DNA.

[b] Derivatives of CFN23, described in the text.

[c] GMI9023 transconjugants in matings with CFN23 derivatives, described in the text.

[d] The construction of this plasmid is described in Materials and Methods.

inserted in the 9.8-kb *Bam*HI fragment that carries one of the *nifH* genes. This strain was called CFN2414 (see Results).

Heat treatment. *R. phaseoli* strains were heated by a modification of the procedure described by Zurkowski (33) to cure *R. trifolii* plasmids. A fresh culture of the bacteria was streaked in YM medium (33) and incubated for 5 days at 37°C. Single colonies were isolated at 30°C and tested for nodulation or antibiotic resistance. To determine the frequency of nonnodulating derivatives, 50 colonies were independently tested on bean plants; to test antibiotic susceptibility, 100 colonies were replicated onto agar plates with and without the antibiotic.

Matings. Matings were done as described by Quinto et al. (26). Tn*5 mob* insertions were isolated from a triparental mating between HB101(pRK2013), HB101(pSUP5011), and CFN23 Rif^r (Table 1).

The mobilization of the Tn*5 mob* insertions in the Sym plasmid was done by using HB101(pJB3JI) (Table 1) as a helper plasmid or with HB101(pRK2013) (Table 1) in a triparental cross. In the matings where Nod$^+$ transconjugants were infrequent, the nodulation of beans was used for selection; transconjugants were purified from nodules and tested again for their nodulation ability. When pJB3JI was used to mobilize plasmids marked with Tn*5 mob*, tetracycline-susceptible transconjugants were isolated to avoid the inheritance of the helper plasmid.

The frequency of CFN23 Sym plasmid transfer is about 10^{-6} per donor cell, but when the Sym plasmid Tn*5 mob* insertions were mobilized by pJB3JI or pRK2013 (Table 1), the frequency of kanamycin-resistant transconjugants was about 10^{-4} per donor cell.

Isolation and manipulation of DNA. Isolation of DNA, radioactive labeling, and Southern blot hybridization were carried out as reported by Quinto et al. (25). *nifH* and Tn*5* probes were those described previously (26). None of these probes hybridized with the total DNA of *A. tumefaciens* GMI9023 (Table 1).

Plasmid visualization. Plasmid visualization was done by the Eckhardt (7) procedure. The Sym plasmid was purified from strain CFN2302 (Table 1) by the Hirsch et al. (9) procedure and banded in a CsCl gradient containing ethidium bromide. The plasmid molecular weight was calculated from the comparison with *R. phaseoli* CFN42 plasmids run in the same agarose gel.

Nodulation. Conditions for nodulation of beans were as reported by Martínez et al. (16).

RESULTS

Instability of the symbiotic properties of *R. phaseoli* CFN23. *R. phaseoli* strains isolated in different regions of Mexico frequently lose their symbiotic phenotype at a high frequency after they are incubated at 37°C, a treatment that has been used to cure *Rhizobium* sp. plasmids (33). One of these strains, CFN23, was studied in detail in this work.

Strain CFN23 lost its symbiotic phenotype at a frequency of 62% after growth at 37°C for 5 days (Table 2). We asked whether the loss of the nodule-forming ability correlated with a loss of the symbiotic (Sym) plasmid, but the plasmid electrophoretic pattern of strain CFN23 was identical to the pattern of several of its nonnodulating (Nod$^-$) derivatives

TABLE 2. Percentage of loss by heat treatment[a]

Strain	% of strains with indicated phenotype		
	Km^s	Sp^s	Nod$^-$
CFN23			62
CFN2315	76		NT[b]
CFN2314	<1		74
CFN2414	<1	50	NT[b]

[a] Growth on petri dishes for 5 days at 37°C. See footnote *a* of Table 1 for abbreviations.

[b] NT, Not determined directly, but all the antibiotic-susceptible derivatives tested were Nod$^-$ and lacked the three *nifH* copies.

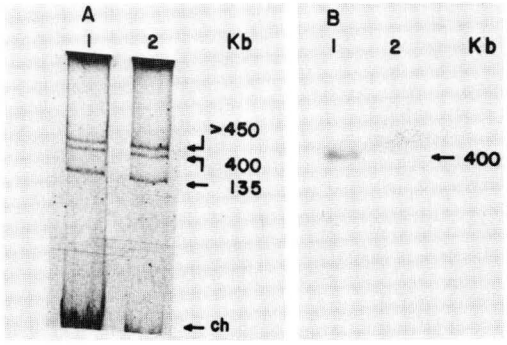

FIG. 1. (A) Plasmid electrophoretic pattern of wild-type and cured strains on 0.7% agarose gels stained with ethidium bromide and (B) hybridization of this plasmid profile with the 0.9-kb *nifH* probe. Lanes: 1, CFN23; 2, CFN2370. Numbers correspond to molecular sizes of plasmids in kb. ch, Chromosomal DNA.

(data not shown). Nevertheless, the loss of the *nifH* genes in all Nod$^-$ derivatives tested was demonstrated by DNA hybridization. An example of this is shown in Fig. 1, which shows the plasmid electrophoretic pattern and the absence of the *nifH* homologous sequences in the CFN23 Nod$^-$ derivatives CFN2370 (Table 1).

Deletion of the Sym plasmid in the CFN23 Nod$^-$ derivatives. We could not detect any change in the molecular weight of the CFN23 Sym plasmid when the three *nifH* genes were lost (Fig. 1). A deletion of up to 50 kb would not be seen in a 400-kb plasmid, so we looked for the presence of the replicon by using the whole Sym plasmid as a probe in hybridization experiments with the plasmid profiles of strains CFN23 and CFN2370 and in Southern blot experiments. We found that strain CFN2370 conserved a plasmid homologous to the CFN23 Sym plasmid (data not shown).

A Tn*5 mob* insertion in the CFN23 Sym plasmid was isolated; the CFN23 derivative with this insertion is called CFN2315. The Sym plasmid location of this Tn*5 mob* insertion is shown in Fig. 2, lanes 2. The frequency of loss of the kanamycin resistance of CFN2315 was determined after heat treatment, and a frequency of kanamycin-susceptible derivatives similar to that of loss of nodulation capacity in CFN23 was found (Table 2). In Fig. 2, lanes 4, the relevant characteristics of one of these kanamycin-susceptible derivatives (CFN2350, Table 1) are shown. By the criteria used, CFN2350 is identical to CFN2370 (Fig. 1, lanes 2). The frequency of kanamycin-susceptible derivatives of CFN2315 when grown at 30°C was 1%; this is a much lower value than at 37°C (Table 2) but sufficiently high to become a problem if CFN23 is used as an inoculant in the field.

When the plasmid marked with Tn*5 mob* in CFN2315 was mobilized to a plasmidless *A. tumefaciens* strain (GMI9023, Table 1), the transconjugants gained the ability to nodulate beans. The nodules made by these transconjugants were similar to those made by CFN2315. Leghemoglobin was present in both instances (data not shown). One of these transconjugants (CFN2302, Table 1) was studied in detail; we found that it contains a single plasmid with the same molecular weight as the CFN23 Sym plasmid, and that *nifH* and Tn*5* probes gave the same hybridization pattern as CFN2315 DNA (Fig. 2, lanes 2 and 3).

Isolation of a stable Tn*5 mob* insertion in the CFN23 Sym plasmid. A stable Tn*5 mob* insertion in the Sym plasmid was isolated in strain CFN2314 (Table 2). We conclude that the Tn*5 mob* insertion in CFN2314 was located in the Sym plasmid, because both Tn*5* and *nifH* probes hybridized with the same plasmid band as in CFN2315 (Fig. 2, lanes 2 and 5).

To show that the Tn*5 mob* insertion in CFN2314 was indeed in the same plasmid as the *nifH* genes (i.e., the Sym plasmid), we determined the genetic linkage of these sequences. We constructed a derivative of CFN2314,

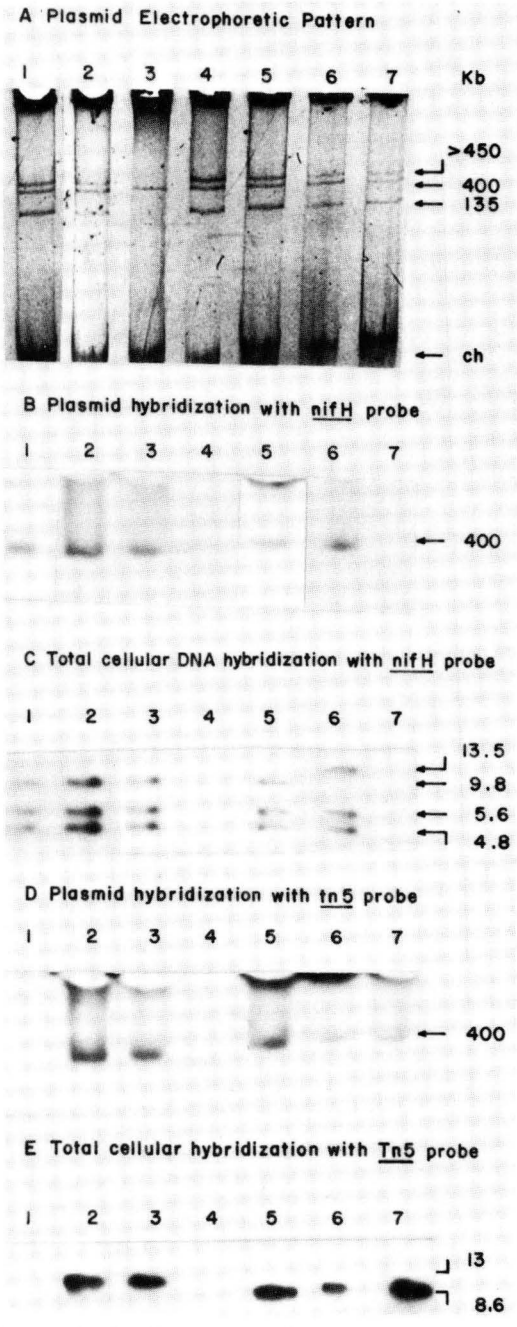

FIG. 2. Hybridization patterns of plasmid profiles and total cellular DNA digests of CFN23 and derivatives: (A) plasmid electrophoretic patterns on 0.7% agarose gels, Southern blot hybridization probed with *nifH* of (B) plasmid electrophoretic patterns and (C) total cellular DNA digested with *Bam*HI, and Southern blot hybridizations probed with Tn*5* of (D) plasmid electrophoretic patterns and (E) total cellular DNA digested with *Eco*RI. Lanes: 1, CFN23; 2, CFN2315; 3, CFN2302; 4, CFN2350; 5, CFN2314; 6, CFN2414; 7, CFN2340. Numbers correspond to molecular sizes of DNA sequences in kb. ch, Chromosomal DNA.

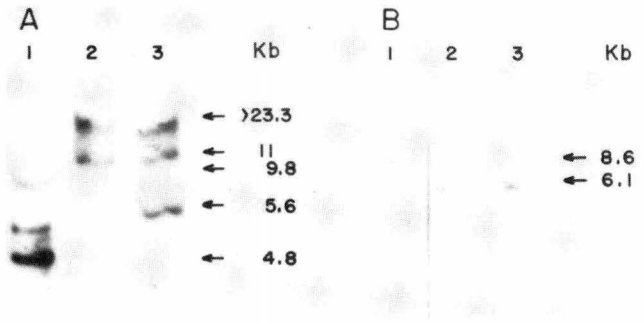

FIG. 3. Southern blot hybridization of CFN2314 and strains showing *nifH* rearrangements. (A) *nifH* was used as the probe, and DNA was digested with *Bam*HI; (B) DNA was digested with *Eco*RI and a Tn*5* probe was used. Lanes: 1, CFN2314; 2, CFN2303; 3, CFN2304. Numbers correspond to the sizes of the hybridizing fragments in kb.

CFN2414, in which spectinomycin resistance determinant was recombined in one of the *nifH* genes (see Materials and Methods; Fig. 2, lane 6), so the presence of this gene could be followed by the antibiotic resistance. Mating experiments were done by using CFN2370 as a recipient; the frequency of transfer of kanamycin resistance from CFN2315 and CFN2314 was about 10^{-6}. The Sym plasmid location of the Tn*5* *mob* insertion in CFN2315 was shown by the contransfer of the kanamycin resistance, *nifH*, and nodule-forming ability when the plasmid was mobilized to *A. tumefaciens* GMI9023 (Fig. 2, lanes 3). If the Tn*5* *mob* insertion in CFN2414 was not located in the Sym plasmid, then the frequency of contransfer of kanamycin and spectinomycin resistances in this strain should be of about 10^{-12}. Among the CFN2370 transconjugants that inherited kanamycin resistance from CFN2414, 76% were also spectinomycin resistant; therefore we conclude that both markers are in the same plasmid.

The spectinomycin resistance of CFN2414 was unstable after heat treatment even though the kanamycin resistance was not (Table 2). In Fig. 2, lanes 7, we show the characteristics of one of the CFN2414 spectinomycin-susceptible derivatives (CFN2340, Table 1). This strain is similar to CFN2370 and CFN2350 but retains the Tn*5* *mob* insertion in the same position as in CFN2314.

Rearrangements of CFN23 Sym plasmid that affect *nifH* position. The *nifH* and Tn*5* *mob* positions in the *A. tumefaciens* transconjugant CFN2302 are the same as in the parent *R. phaseoli* strain, CFN2315 (Fig. 2, lanes 2 and 3), and both strains are able to nodulate beans. When the Sym plasmid was mobilized from CFN2314 to *A. tumefaciens* GMI9023, the ability to nodulate beans was not inherited by 20 independent transconjugants tested in plants. Total DNA from one of these transconjugants (CFN2303) was hybridized with *nifH* and Tn*5* probes, and a new structure of the Sym plasmid was found in which the position of the *nifH* genes and Tn*5* was altered (Fig. 3, lanes 2).

CFN2303 was unable to nodulate beans, even though it contains at least part of the Sym plasmid as judged by the presence of *nifH* and Tn*5* *mob*. We decided to mobilize the CFN2303 plasmid to other genetic backgrounds to determine whether the nodulation ability might be regained. Unexpectedly, we found that the nodulation ability could be regained when CFN2303 was used as donor in mating experiments, although no transfer of genetic material was detected. Both CFN2303 and *A. tumefaciens* C58C1 are Nod$^-$ (Table 1); a mixture of these bacteria was used to inoculate beans and to isolate an Nod$^+$ transconjugant; nodules were found, but the bacteria isolated from these nodules had the genetic markers of strain CFN2303 and not those of C58C1. Total DNA isolated from one of CFN2303 Nod$^+$ derivatives (CFN2304, Table 1) was digested and hybridized with the *nifH* and Tn*5* probes; the hybridization pattern was different from that of CFN2303 (Fig. 3, lanes 3). Another independent CFN2303 Nod$^+$ derivative showed the same *nifH* structure as CFN2304 (data not shown).

DISCUSSION

We have identified the Sym plasmid of *R. phaseoli* CFN23 and shown that it contains the information that enables a plasmidless *A. tumefaciens* strain to form nodules in association with beans.

This Sym plasmid participates in recombination events which give rise to different molecular structures. The changes in structure modify the symbiotic phenotype of the bacteria. This high frequency of genetic rearrangements that affect the symbiotic information of *R. phaseoli* could make this bacteria a very adaptable symbiont.

We found that the most frequent genetic rearrangement is represented by the Nod$^-$ derivatives of CFN23. In these derivatives the three *nifH* genes are lost, but the Sym plasmid is not segregated, and the molecular weight of the plasmid is not appreciably affected (Fig. 1 and 2). These data could be explained as resulting from a small deletion which was not detected in the plasmid profile. Nevertheless, there is evidence in another *R. phaseoli* strain (CFN42) that the three *nif* regions present in the CFN42 Sym plasmid are not closely linked, and a deletion that would remove all the nitrogenase structural genes represents a loss of approximately 120 kb of DNA (R. Palacios, personal communication). We have evidence that the Nod$^-$ derivatives of CFN23 have not only *nifH* but also all of the structural genes of the nitrogenase; if the CFN42 Sym plasmid structure is conserved in CFN23, the most frequent rearrangement found in this molecule would require a large deletion of the plasmid and the substitution for this DNA by other DNA sequences so that the molecular weight of the plasmid could be conserved. We are carrying out experiments to test this hypothesis of deletion and substitution of DNA.

The different frequences at which kanamycin resistance was lost in strains CFN2315 and CFN2314 (Table 2) reflects the frequency at which the site of insertion of the transposon in each strain participates in the Sym plasmid rearrangement described above, i.e., the Tn*5* *mob* insert in CFN2315 was deleted when *nifH* genes were lost, but the one in CFN2314 was conserved in the deleted Sym plasmid.

The Sym plasmid structure in strain CFN2303 represents another genetic rearrangement. In this molecule, the positions of the *nifH* genes and of the Tn*5* *mob* insertion are different from those in the plasmid of the parent strain CFN2314, although this alteration cannot be explained by a single recombination event. It might be the product of multiple events which generate unstable intermediates. We do not know whether the CFN2303 Sym plasmid structure is formed during the conjugal transfer of this plasmid or whether it represents a small proportion of the plasmids present in the CFN2314 population that are more frequently transferred. If the second possibility is true we will be able to isolate a CFN2314 derivative that would be Nod$^-$ and retain the *nifH* genes. The Sym plasmid structure in CFN2303 could be also a product of rearrangements in *A. tumefaciens*. We are now looking for its presence in different rhizobial backgrounds.

The bacteria harboring the CFN2303 plasmid are unable to nodulate beans, but Nod^+ derivatives can be isolated in which the position of the *nifH* genes is altered (strain CFN2304, Fig. 3). This suggests that the Sym plasmid structure of strain CFN2303 does not allow the expression of the nodulation genes, although this information is present in the plasmid. The Nod^+ derivatives of strain CFN2303 could only be obtained when this strain was used as donor in a genetic cross, even though no transfer of genetic information was detected.

All of the rearranged Sym plasmid derivatives that hybridize with the *nifH* probe have more than one copy of this gene (CFN2303 and CFN2304, Fig. 3), and we found that the different arrangements of this gene are very similar to those present among wild-type *R. phaseoli* isolates (16). We conclude from these data that the *nifH* reiteration is a characteristic of *R. phaseoli* Sym plasmids and that the Sym plasmid rearrangements found under laboratory conditions also happen in nature.

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Formation of *Rhizobium phaseoli* Symbiotic Plasmids by Genetic Recombination.

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ABSTRACT

A hybrid *Rhizobium phaseoli* symbiotic plasmid was detected; this plasmid, pSym2342, is the product of the recombination between parts of the symbiotic plasmid of two independent *R. phaseoli* strains (CFN42 and CFN23 strains). The genetic information from CFN42 strain was part of an R' (pRS10). The hybrid symbiotic plasmid (pSym2342) can be transfered to *Agrobacterium tumefaciens* and confers the ability to nodulate beans. We detected the formation of another symbiotic plasmid (pSym4215) by the recombination between pRS10 and an *R. phaseoli* CFN42 indigenous plasmid (p42e); plasmid p42e was thought to be unrelated to the CFN42 symbiotic plasmid. The newly formed symbiotic plasmid (pSym4215) is very similar to the hybrid plasmid pSym2342. These data are discussed with regard to the stability of *Rhizobium phaseoli* symbiotic information.

INTRODUCTION

The *Rhizobium* species comprises the gram negative soil bacteria that nodulate legume roots and fix nitrogen in these nodules, which is then assimilated by the plant.

The genetic information for the symbiotic functions of fast growing *Rhizobia* is plasmid encoded (6, 8, 9, 14); the plasmid that carries the plant species specificity for nodulation and the structural genes of the nitrogenase enzyme (*nif* genes) is called symbiotic plasmid (pSym) (7).

Rhizobium phaseoli is a fast growing *Rhizobium* that has a peculiar genome, as it contains many reiterated DNA sequences (5). In some *R. phaseoli* strains, genetic rearrangements that modify its symbiotic phenotype have been found (20), this genetic plasticity may be related to the presence of reiterate DNA sequences, as shown for *Halobacterium hallobium* (16) and *Streptomyces* species (1).

The symbiotic information of different *Rhizobium phaseoli* strains can be arranged in different ways; some *R. phaseoli* strains loose their ability to nodulate beans at a high frequency due to a deletion affecting their pSym (20) where other strains contain symbiotic plasmid that are not deleted (Soberón-Chávez G. and Nájera R. manuscript submitted for publication). In strain CFN42 some genes involved in its nitrogen fixing ability are

encoded in plasmids different from its pSym (12) whereas in CFN 23 strain the DNA sequence homologous to one of these CFN42 genes is contained in the symbiotic plasmid (see RESULTS).

The deletion affecting the pSym of CFN23 strain has been described, the deleted pSym lacks the three copies of the nitrogenase reductase gene (*nifH*) and the bacteria is no longer able to nodulate beans; the extent of this pSym deletion has been evaluated to at least a 100kb of DNA, but paradoxically it cannot be detected by a change in the molecular weight of the plasmid (20).

To define the extent of genetic information necessary to complement the CFN23 pSym deletion, we isolated a series of R's which complemented for bean nodulation and nitrogen fixation a strain carrying the CFN23 deleted symbiotic plasmid (ΔpSym23).

We describe here the formation of two symbiotic plasmids by genetic recombination, these data are discuss with regard to the stability of *R. phaseoli* symbiotic information and its transfer between bacteria.

MATERIALS and METHODS

Bacterial strain and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Growth conditions PY medium (13) was used to grow *R. phaseoli* strains, unless otherwise stated. *A. tumefaciens* and *E. coli* strains were grown in LB medium (11). *R. phaseoli* and *A. tumefaciens* strains were grown at 30°C and *E. coli* strains at 37°C. The antibiotic concentrations used were as follows: ampicillin 20µg/ml, gentamycin 40µg/ml, Kanamycin 60µg/ml, rifampicin 30µg/ml, streptomycin 200µg/ml and tetracycline 10µg/ml.

Heat treatment *R. phaseoli* strains were heated by a modification of the proceedure described by Zurkowski (22) to cure *R. trifolii* plasmids. A fresh culture of the bacteria was streaked in YM medium (21) and incubated for 5 day at 37°C, single colonies were isolated at 30°C and tested for antibiotic resistance; 100 colonies were replicated onto agar plates with and without kanamycin or tetracycline.

Construction of pSM204. Total DNA of strain CFN2013 (12) was digested with the EcoRI endonuclease and ligated to the EcoRI site of pBR329 as described by Quinto *et al* (17), the recombinant plasmids were transformed in strain HB101 (Table 1); the kanamycin and ampicilin resistant and cloramphenicol

sensitive transformants were selected, pSM204 plasmid is a recombinant plasmid thus selected which contains an insert of 8.6kb (data not shown).

Isolation and Manipulation of DNA. Isolation of DNA, radioactive laveling and Southern blot hybridization were carried out as reported by Quinto *et al* (17) the *nifH* probe was described previously (10).

Plasmid visualization. Plasmid visualization was done by the procedure described by Eckhardt (4).

Nodulation Assay. Bean nodulation was assay as described by Martínez *et al* (10).

RESULTS

pRS10 Selection. The promiscuos plasmid pJB3JI (Table 1) was transfered to the *R. phaseoli* strain CFN2201 (18) (Table 1); strain CFN2201 carries a *Tn5* fragment inserted in one of the genes coding for the nitrogenase reductase (*nifH*), so when the kanamycin resistance is transfered to other bacteria the transfer of the *nifH* sequence is selected. Kanamycin and tetracycline resistant *E. coli* colonies were selected from a mating between strains CFN2201 (pJB3JI) and HB101 (Table 1); the symbiotic plasmid of strain CFN2201 (pSym2201, Table 1) cannot be transfered to *E. coli*, so the resulting *E. coli* transconjugants contain the *R. phaseoli* information forming part of an R'. These R's were transfered to *R. phaseoli* strain CFN2370 (Table 1), which is a CFN23 derivative unable to nodulate beans due to a deletion of its pSym; the *R. phaseoli* transconjugants were tested for bean nodulation.

Plasmid pRS10 is an R' thus selected that complements strain CFN2370 (Table 1) for bean nodulation and nitrogen fixation, this plasmid contains ≈100kb of strain CFN2201 DNA (data not shown) in which the three *nifH* copies are included (Fig. 1).

We determined that pRS10 did not contain all the symbiotic information coded in pSym2201 plasmid by the independent transfer of both plasmids to an *Agrobacterium tumefaciens* plasmid-

less strain (GMI9023, Table 1); GMI9023(pSym2201) nodulated beans but GMI9023(pRS10) did not.

Formation of the hybrid symbiotic plasmid pSym2342. The symbiotic information of *R. phaseoli* CFN23 strain is lost at a high frequency due to a deletion affecting its symbiotic plasmid which removes the three copies of genes coding for the nitrogenase (20); when *Tn5* is inserted in certain regions of this plasmid, as it is in CFN2315 strain (Table 1), the kanamycin resistance is lost at a high frequency (Table 2) and the kanamycin sensitive derivatives loose the ability to nodulate beans and lack *nifH* genes (20).

The symbiotic information of *R. phaseoli* strain CFN42 is very stable, so a *Tn5* inserted in its symbiotic plasmid is seldom lost, as shown in Table 2. pJB3JI plasmid is very stable both in CFN23 and CFN42 backgrounds (Table 2).

We determined the stability of plasmid pRS10 in strain CFN2370, and found that tetracycline resistance was stably mantained while kanamycin resistance was lost at a high frequency, (Table 2); these data suggest that plasmid pRS10 was being dissociated in strain CFN23 background. The plasmid electrophoretic profile of CFN2370 strain carrying pSR10 plasmid shows a plasmid of pJB3JI molecular weight (52kb) and one of pSym2201 plasmid size (300Kb) but the intact pRS10 plasmid was not detected (Fig.2).

Kanamycin sensitive and tetracycline resistant deriva-

tives of CFN2370 strain carrying pRS10 loose the plasmid of the pSym2210 size and conserve the pJB3JI plasmid band; these derivatives are unable to nodulate beans and lack homology to the *nifH* probe (data not shown). These data suggest that the CFN2201 genetic information carried in pRS10 plasmid was included in the 300kb plasmid. We show that the 300kb plasmid was a symbiotic plasmid by the following criteria: kanamycin resistance was transfered from CFN2370(pRS10) to strain GMI9023 (Table 1), which is a plasmidless *Agrobacterium tumefaciens* strain, tetracycline sensitive A. *tumefaciens* transconjugants were found, this transconjugants nodulate beans and contained a single plasmid of 300kb (Fig. 3), this hybrid plasmid with part of the CFN2201 information and part of the information of CFN2370 strain is called pSym2342.

CFN2013 is a mutant strain derived from CFN42 strain with a reduced ability to fix nitrogen. The CFN2013 mutation is caused by a *Tn5* insertion in the plasmid p42*e*, which is a 400kb CFN42 indigenous plasmid different from the CFN42 pSym(p42*d*) (12). The CFN2013 mutation was cloned (see MATERIALS and METHODS) and the recombinant plasmid carrying this mutation (pSM204) was used as probe to look for its presence in CFN23 strain: we found, that strain CFN23 h as homology with pSM204 and that the homologus sequence was carried in its pSym (Fig. 4).

In order to show that plasmid pSym2342 was derived from

the deleted CFN23 plasmid, we transfered pSym2342 to strain GMI9023 and determined that the homology with pSM204 was cotransfered (Fig. 5 lane 5); plasmid pSym2201 does not share any homology with pSM204 besides *Tn5* (Fig. 5 lane 5).

Formation of the symbiotic plasmid pSym4215. An *R. phaseoli* CFN42 derivative cured of the entire symbiotic plasmid has been isolated (15); plasmid pRS10 was transfered from *E. coli* strain HB101 to this cured strain (CFN2001, Table 1), we found that pRS10 was also dissociated in CFN2001 background by the following criteria: kanamycin resistance is lost at a high frequency in CFN2001(pRS10), while tetracycline resistance is not (Table 2), and both the pJB3JI band and that of 300kb are apparent, (Fig. 3) the kanamycin sensitive derivatives loose the 300kb plasmid and conserve that of pJB3JI molecular weight (Fig. 3); kanamycin sensitive derivatives are unable to nodulate beans and lack homology with *nifH* probe (data not shown).

A new symbiotic plasmid (pSym4215) was identified by analizing the kanamycin resistant, tetracycline sensitive transconjugants from the mating between *R. phaseoli* CFN2001(pRS10) and GMI9023 *A. tumefaciens* strain, these transconjugants can form nodules on beans and contain a single plasmid (Fig. 3).

To determine if plasmid p42e was involved in the formation of pSym4215 plasmid we looked for the homology of plasmid pSym4215 with plasmid pSM204 which was known to hybridize with plasmid p42e, we found that pSym4215 has homology with pSM204

besides the *Tn5* hybridizing band (Fig 5, lane 4).

Plasmids pSym2201 and pSym4215 have the same molecular weight, and share the symbiotic information carried in plasmid pRS10, but the frequency of the kanamycin resistance loss in each plasmid is very different (Table 2); plasmid pSym4215 is lost at a rate similar to that of deletion affecting the symbiotic plasmid of CFN23 strain.

DISCUSSION

We described the formation of two symbiotic plasmids pSym2342 and pSym4215 by genetic recombination, the frequency of formation of these plasmids is so high that in an *R. phaseoli* background we could not detected the presence of pRS10, one of the plasmids participating in the symbiotic plasmids formation.

The size of the DNA sequence necessary to complement strain CFN2370 for nodulation and nitrogen fixation is ≃100kb the size of the pSym2201 DNA carried in pRS10, so we suppose that the deletion of CFN23 symbiotic plasmids about 100kb. CFN42 symbiotic plasmid has 100kb less DNA than CFN23 symbiotic plasmid; this different in size is conserved between pSym2342, and CFN23 symbiotic plasmid. The deletion affecting the CFN23 symbiotic plasmid could be of ≃200kb if the 100kb of "extra" DNA in CFN23 symbiotic plasmid are located in the region of the plasmid where *nifH* genes are located.

We elaborate a model to explain the loss of 200kb of DNA without a change in the molecular weight of CFN23 symbiotic plasmid, this model is based in the recombination of two copies of this plasmid (Fig. 6); the recombination of these plasmids could result in the formation of two plasmids; one plasmids would have two copies of the part of the Sym plasmid which includes *nif* genes and some nod genes, this plasmid should be unable to

replicate in *Rhizobium*; and another plasmid with two origins of replication lacking *nif* genes and some *nod* genes; the molecular weight of both plasmid resulting from the recombina- should be 400kb, the same molecular weight as the entire Sym plasmid. We are now involved in the evaluation of this model by analysing the structures of ΔpSym23 plasmid. We have isolated a CFN23 mutant with enhanced homologous genetic recombination; the symbiotic plasmid of this mutant is deleted more frequently than that of the wild type CFN23 strain (Soberón-Chávez G. and Nájera R. unpublished results) this suggest that recombination is indeed involved in the deletion of this pSym.

Plasmids pSym2342 and pSym4215 are lost at a rate similar to the deletion affecting CFN23 symbiotic plasmids, the po s ibility exists that a similar mechanism of deletion-substi- tution of DNA as shown in Fig. 6 is involved in the loss of these plasmids; the recombination of two pSym2342 molecules, will result in the segregation of a 200kb plasmid carring the *nif* genes and the permanence of a plasmid identical to ΔpSym23 plasmid. We think that the high frequency in which the plasmids pSym2342 and pSym4215 are formed, their instability, and the involvement of ΔpSym23 plasmid in the formation of plasmid pSym 2315, are indirect evidences that support this model of plasmid recombination and segregation.

CFN42 indigenous plasmid p42e is very similar to plasmid

ΔpSym23 both have the same molecular weight, are very stable (20, 5), contain homologous sequences (Fig. 5) and as shown here, both participate in the formation of a symbiotic plasmid. We think that CFN42 strain contains a deleted symbiotic plasmid which could be derived from an "unstable" *R. phaseoli* pSym, and, coexisting in the same cell, a different type of symbiotic plasmid that is not deleted at a significant rate.

The only detectable difference between the strains CFN 2201 and CFN2001(pSym4215) is the different stability of their symbiotic properties which may be due to the differences in their symbiotic plasmids. These strains are isogenic, with the exception of part the original CFN42 symbiotic plasmid, which is absent from CFN2001(pSym4215); from the comparison of these *R. phaseoli* strains we conclude that the stability of the symbiotic phenotype of *Rhizobium phaseoli* is determined by the difference in the structure of the symbiotic plasmids and not from differences in the DNA metabolism of different *R. phaseoli* strains.

It is difficult to explain that *R. phaseoli* strains which loose their ability to nodulate beans at a high frequency are isolated from bean nodules, the loss of the symbiotic information of these strains has to be compensated by a high frequency of reincorporation of this information; our finding of the high frequency of formation of symbiotic plasmids by recombination

may reflect on the ability of the bacteria that contained a deleted symbiotic plasmid to reincorporate the information necessary to nodulate beans effectively.

A necessary condition for the reincorporation of the symbiotic information by bacteria that once were *R. phaseoli*, is that this information is transfered between bacteria in soil at a significant rate, this transfer has not been detected in laboratory conditions, but we are now studying the transfer of the symbiotic information when *R. phaseoli* is inoculated to soil.

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Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant characteristics[a]	Source of reference
BACTERIA:		
R. phaseoli CFN23	Nod$^+$ *nifH*$^+$. Looses the ability to nodulate beans at a high frequency.	(20)
R. phaseoli CFN2315	Nod$^+$ *nifH*$^+$. *Tn5mob* inserted in the symbiotic plasmid. Rif^R Km^R. Derivative of CFN23 strain.	
R. phaseoli CFN2370	Nod$^-$ Δ*nifH*. Contains a deleted symbiotic plasmid. Str^R. Derivative of CFN23 strain.	(20)
R. phaseoli CFN42	Nod$^+$ *nifH*$^+$. It's nodule forming ability is very stable.	(17)
R. phaseoli CFN2201	Nod$^+$ *nifH*$^+$. Contains a *Tn5* fragment inserted in one *nifH* copy. St^R Km^R. Derivatives of CFN42 strain.	(18)
R. phaseoli CFN2001	Nod$^-$ *nifH*$^-$. Lacks the entire CFN42 symbiotic plasmid. Rif^R. Derivative of CFN42 strain.	(15)
R. phaseoli CFN2026	Mutant strain unable to fix nitrogen derived from CFN42 strain, mutation is caused by the insertion of *Tn5* in p42*e* . Km^R Sm^R.	(18)
A. *tumefaciens* GMI9023	Vir$^-$ (plasmid less). St^R Rif^R.	(19)
E. coli HB101	*recA hsdR hsdM*. Str^R.	(2)
PLASMIDS:		
PJB3JI	Able to replicate in *Rhizobia* and *Enterobacteria* Tc^R Gm^R Km^S	(3)
pSym2201	Symbiotic plasmid of CFN2201 strain, *NifH*$^+$, contains a *Tn5* fragment inserted in one *nifH* copy. Km^R.	(17)
pRS10	R' by cointegration of pJB3JI and part of pSym2201. *nifH*$^+$ Tc^R Gm^R Km^R.	This work
pSym23	Deleted symbiotic plasmid of CFN23 strain. Δ*nifH*.	(20)
pSym2342	*R. phaseoli* symbiotic plasmid formed by recombination of Δ pSym23 and pRS10 *nifH*$^+$ km^R.	This work
p42*e*	CFN42 strain indigenous plasmid.	(12)
pSym4215	*R. phaseoli* symbiotic plasmid formed by recombination of CFN42*e* indigenous plasmid and pRS10. *nifH*$^+$ Km^R.	This work
pSM204	Recombinant pBR329, with a DNA inserted which includes CFN2026 (11) mutation Km^R Ap^R, Tc^R, Cm^S.	This work

[a] Abreviations used: Nod, ability to nodulate beans, Vir, tumor forming ability; *mob* DNA sequence which determines the ability to be mobilized by some transferable plasmids; *nifH*, nitrogenase reductase gene. Resistance to ampicilim (Ap^R) streptomycin (Str^R), rifampicin (Rif^R), kanamycin (Km^R), tetracycline (Tc^R) and gentamicin (Gm^R), and suceptibility to kanamycin (Km^S) and cloramphenicol (Cm^S) are indicated. *recA* mutants are unable to recombine, *hrdR* and *hsdM* mutants are unable to modify or.

Table 2. Percentage of loss by heat treatment[a]

Strain	% of derivatives sensitive to;	
	Km	Tc
CFN2315	85	
CFN2370(pJB3JI)		<1
CFN2370(pRS10)	90	<1
CFN2201	<1	
CFN2001(pJB3JI)		<1
CFN2001(pRS10)	77	<1

[a] Growth on petri dishers for 5 days at 37°C. See foot note a of Table 1 for abreviations.

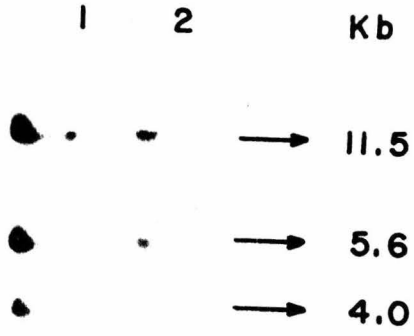


Figure 1

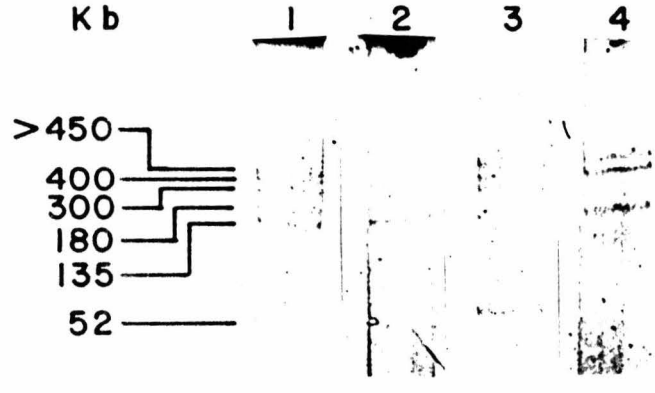


Figure 2

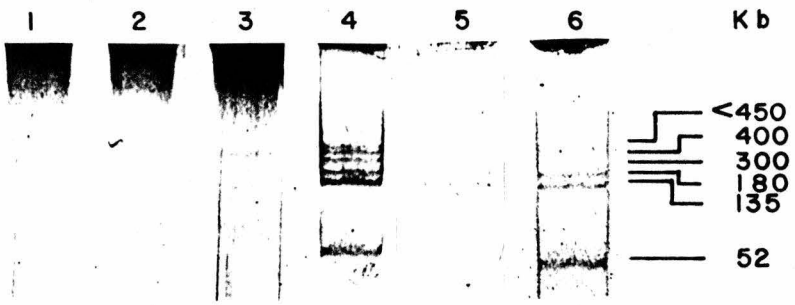


Figure 3

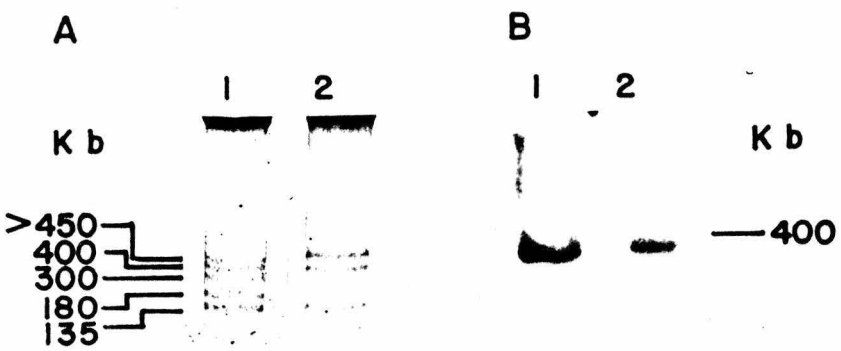


Figure 4

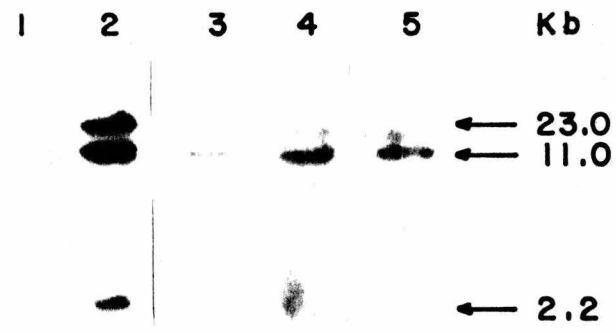
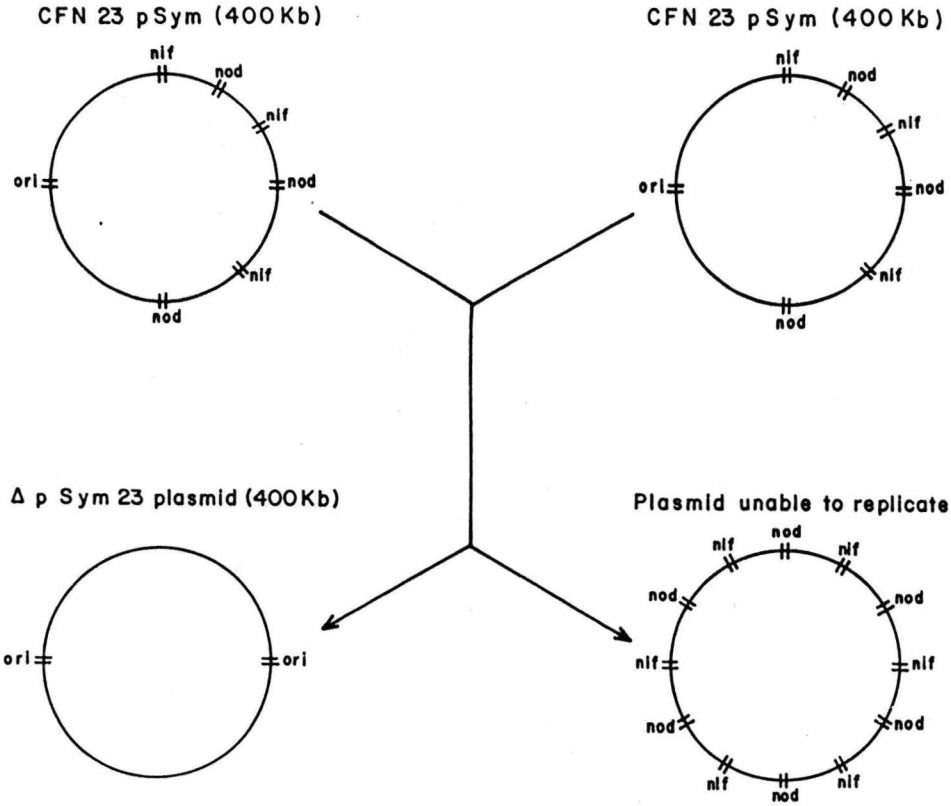


Figure 5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nif
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CFN 23 pSym (400 Kb)
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Δ p Sym 23 plasmid (400Kb)
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Plasmid unable to replicate
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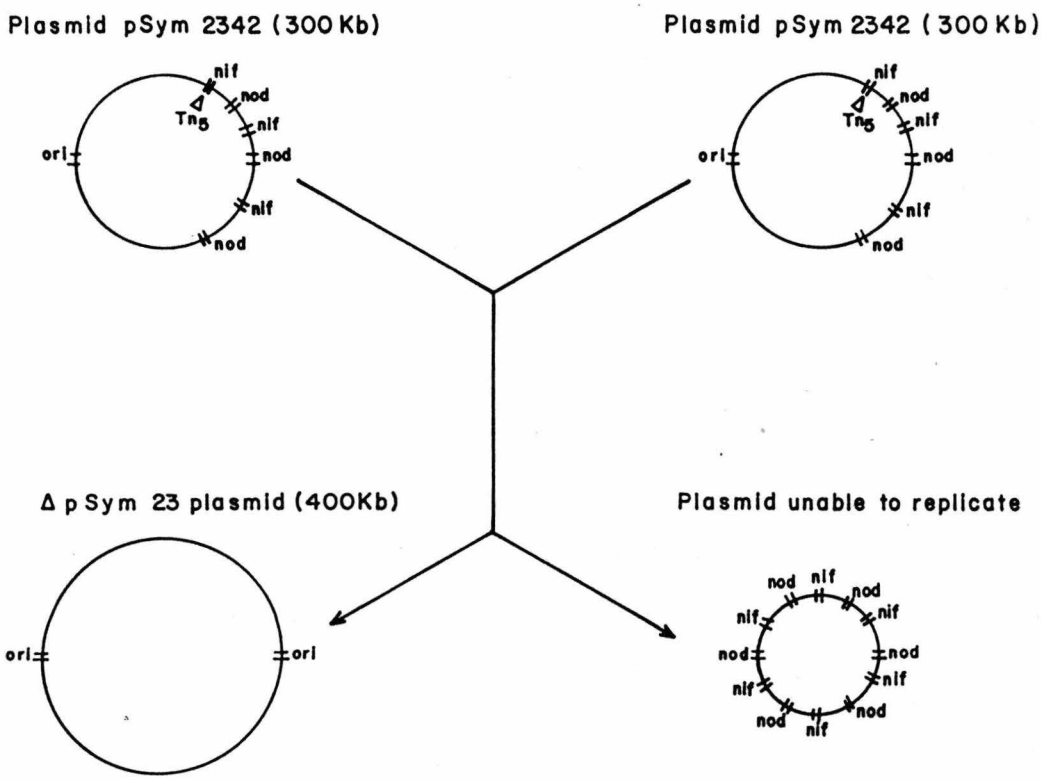
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Δ p Sym 23 plasmid (400Kb)
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Plasmid unable to replicate
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Figure 6

FIGURE LEGENDS

Figure 1. *nifH* hybridization pattern of total cellular DNA digested with BamHI endonuclease. Lanes correspond to the following strains 1) *R. phaseoli* CFN2201 2) *E. coli* HB101(pRS10) 3) *E. coli* HB101. Numbers correspond to molecular weights of the DNA hybridizing fragments in kilobases (kb).

Figure 2. Plasmid electrophoretic pattern on 0.7% agarose gels stained with ethidium bromide. Lane correspond to the following strains (1) CFN2201, (2) CFN2370 (3) CFN2370(pRS10) (4) CFN23. Numbers correspond to molecular weights of the plasmids in kilobases (kb).

Figure 3. Plasmid electrophoretic pattern on 0.7% agarose stained with ethidium bromide. Lanes correspond to the following strains: (1) GMI9023(pSym2201) (2) GMI9023(pSym2342) (3) GMI9023(pSym 4215) (4) CFN2001(pRS10) (5) CFN2001 (6) CFN2001(pRS10) kanmycin sensitive derivative.

Figure 4. (A) Plasmid electrophoretic pattern on 0.7% agarose gels stained with ethidium bromide and (B) its hybridization with pSM204 as probe. (1) CFN42 strain (2) CFN23 strain.

Figure 5. pSM204 hybridization versus total cellular DNA digested with BamHI endonuclease. Lanes correspond to the following strain (1) GMI9023 (2) CFN2201 (3) GMI9023(pSym2201) (4) GMI9023(pSym4215) (5) GMI9023(pSym2342).

Figure 6. Schematic representation of a model to explain the deletion affecting the symbiotic plasmid of strain CFN23, based in the recombination of two of these plasmids resulting in the loss of the symbiotic information without a change in the molecular weight of the plasmid. (B) Scheme of the model to explain the high frequency of loss of pSym2342 based on the recombination of two molecules of this symbiotic plasmid.

Title: Indigenous Soil Bacteria which Can Nodulate Beans and Fix Nitrogen by Complementation with a *Rhizobium phaseoli* Symbiotic Plasmid.

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ABSTRACT

Indigenous soil bacteria lacking symbiotic information were isolated; these bacteria share some *Rhizobium phaseoli* features, and can be complemented to form nitrogen fixing nodules in beans by an *R. phaseoli* symbiotic plasmid; these indigenous soil bacteria contain glutamine synthetase II activity which is characteristic of the *Rhizobiacea*.

Rhizobium and Agrobacterium are related soil bacteria that belong to the same taxonomical family, i.e. the Rhizobiaceae. The condition sine qua non for a bacterium to be classified as Rhizobium, is its ability to form nitrogen fixing nodules in the roots of legumes. Among the genera Rhizobium the species are determined by the legume that the different bacteria are able to nodulate, for example the bacteria isolated from clover nodules are R. trifolii, those taken out from alfalfa nodules are R. meliloti and the ones isolated from bean nodules are R. phaseoli.

Rhizobium classification does not necessarilly correlate with the taxonomic relationships of these bacteria, for example Rhizobium phaseoli is a very heterogenic group as judged by the diversity of their protein profiles[4] and Rhizobium japonicum englobes very diverse bacteria that can share very little total DNA homology[2] (6).

The diversity among the Rhizobium species that are fast growers can be explained because in these bacteria the specificity for nodule formation and the genes involved in the nitrogen fixation process (nif genes) are plasmid encoded[7,10,16,18]. This being the case here, fast growing Rhizobium species are classified by a plasmid coded phenotype[2]. The bacteria that does not contain the plasmid that codes for the symbiotic functions (Sym plasmid) cannot be included in the taxonomic classification.

The study of the pre-*Rhizobium*, that is to say, the bacteria with *Rhizobium* type characteristics, but that do not contain the Sym plasmid, would be very helpfull to the study of *Rhizobium* biology. The life cycle of these bacteria in soil, the influence of different plants on its growth, the frequency of symbiotic information transfer, the effect which this information has on the bacteria-plant interaction and their relation with other soil inhabitants, are some of the important questions that can be studied using a pre-*Rhizobium* strain. On the other hand, the molecular genetic studies of the symbiotic process could be much clearer in a defined genetic background in which the symbiotic information can be transfered and expressed.

Specific nodulation has been complemented in *Agrobacterium tumefaciens* genetic background by several Sym plasmids, but in no case were the nodules able to fix nitrogen (8, 9, 11, 23).

We described here the isolation of indigenous soil bacteria wich could be considerer as pre-*Rhizobium phaseoli* strains based on some of the cammon features that these bacteria share with *Rhizobium phaseoli* strains and in their ability to fix nitrogen in association with beans when they inherit an *R. phaseoli* symbiotic plasmid.

Nalidixic acid resistant bacteria were isolated from bean rhizosphere soil; this resistance marker was chosen because all our *Rhizobium phaseoli* strains (more than a hundred and fifty) are naturally resistant to high levels (80µg/ml) of nalidixic acid. Bean rhizosphere soil was used because the growth of bacteria appart from the specific *Rhizobium* is known to be stimulated in the legumes rhizosphere (14, 20).

The bacteria able to grow in Luria Broth (13) were discarded, as the inability to grow in this medium is another general feature of *R. phaseoli* strains. Bacteria were then streaked in Yeast Mannitol medium (24), and those that made gummy colonies in three to four days at 30°C and did not grow at 37°C were selected. To be able to further manipulate these bacteria, we obtained spontaneous streptomycin (200µg/ml) resistant mutants and discarded all those who were naturally resistant to kanamycin (60µg/ml).

At this stage we checked that all the strains were unable to nodulate beans (12) and lacked total DNA homology with *Rhizobium phaseoli* nitrogenase reductase gene (*nifH*) (19).

A *Rhizobium phaseoli* Sym plasmid, that of strain CFN2315 (22), was transfered to the soil bacteria thus selected, and the transconjugants were tested for their ability to nodulate beans and to fix nitrogen in this association, we found that two strains

CFN2500 and CFN4400 (Table 1) were able to form nitrogen fixing nodules when they inherit CFN2315 Sym plasmid (CFN2515 and CFN4415 Table 1), the inability of CFN2500 and CFN4400 to grow in LB medium is shown in Table 1.

We compared the nitrogen fixing ability of CFN2515 and CFN4415 with that of CFN2302 (22), which is an *Agrobacterium* strain that contains CFN2315 Sym plasmid (Table 1), and we were able to show that the *Rhizobium phaseoli* like bacteria, CFN2515 and CFN4415, present nodules with levels of nitrogenase activity which are similar to those made by a true *Rhizobium phaseoli* strain (CFN2315), while the nodules made by CFN2302 presented a much lower nitrogenase activity meassured by the acetylene reduction assay (30) of whole bean roots (Table 1).

The presence of two glutamine synthetase isozymes is characteristic of the *Rhizobiacea* (4), one of these two enzymes glutamine synthetase II is particular to this family. We show that both CFN2500 and CFN4400 present glutamine synthetase II activity at similar levels to those found in the CFN2315 and CFN2302 strains (Table 1). 3-ketolactose production is characteristic of the genera *Agrobacterium* (3). CFN2500 and CFN4400 as well as *Rhizobium phaseoli* CFN2315 lack-3-ketolactase activity (Table 1).

CFN2500 and CFN4400 are different bacteria as evidence

by their different duplication time both in rich PY medium (15) and in minimal Y medium (1) (Table 1), and their different plasmid electrophoretic pattern (5) (Fig. 1); both isolates present a high molecular weight plasmid whose functions is unknow, many *Rhizobium* isolates present high molecular weight plasmids different from the symbiotic plasmid (16).

Thus we have described the isolation of soil bacteria that can be complementated for bean nodulation and nitrogen fixation ability by a *Rhizobium phaseoli* Sym plasmid. The use of symbiotic plasmids from different *Rhizobium* species to complement effective nodulation of CFN2500 and CFN4400 will show how tight is the relation between these soil bacteria and the symbiotic information that they express. The study of the dynamics of *Rhizobium* symbiotic information in soil, using these "pre-*Rhizobium*" strains, will give light in regard to the importance of the rearrangements of plasmids and chromosomes in the stablishment and perpetuation of the symbiosis between *Rhizobium* and legumes.

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Table 1. Characteristics of soil isolates CFN2500 and CFN4400 compared to *Rhizobium phaseoli* CFN2315 and *Agrobacterium tumefaciens* strain CFN2302.

Strain	Relevant Characteristics[a]	Duplication time (hrs) PY	LB	Y	Bean nodulation	Nodules nitrogenase activity %	Glutamine synthetase II specific activity	3-ketolactose production
CFN2315	*R.phaseoli* CFN23 strain with *Tn5Mob* inserted in the Sym plasmid, Rif^R Km^R (22).	3	<10	3.5	yes	100	0.54	no
CFN2302	A. *tumefaciens* strain GMI9023 with the CFN2315 Sym plasmid Rif^R, Str^R, Km^R (22).	2.5	2.5	3	yes	7	1.89	yes
CFN2500	Soil Isolate, Str^R, This work	3	<10	4	no		1.63	no
CFN2515	CFN2500 with the CFN2315 Sym plasmid, Str^R, Km^R.	2.5	<10	4	yes	120	N.D.[a]	N.D.[a]
CFN4400	Soil Isolate, Str^R. This work	6	<10	6.5	no		0.58	no
CFN4415	CFN4400 with the CFN2315 Sym plasmid, Str^R, Km^R. This work.	6.5	<10	6	yes	95	N.D.[a]	N.D.[a]

[a]Abreviations used are: Rif^R: rifampicin resistance, Km^R: kanamycin resistance, Str^R: streptomycin resistance. N.D. not done.

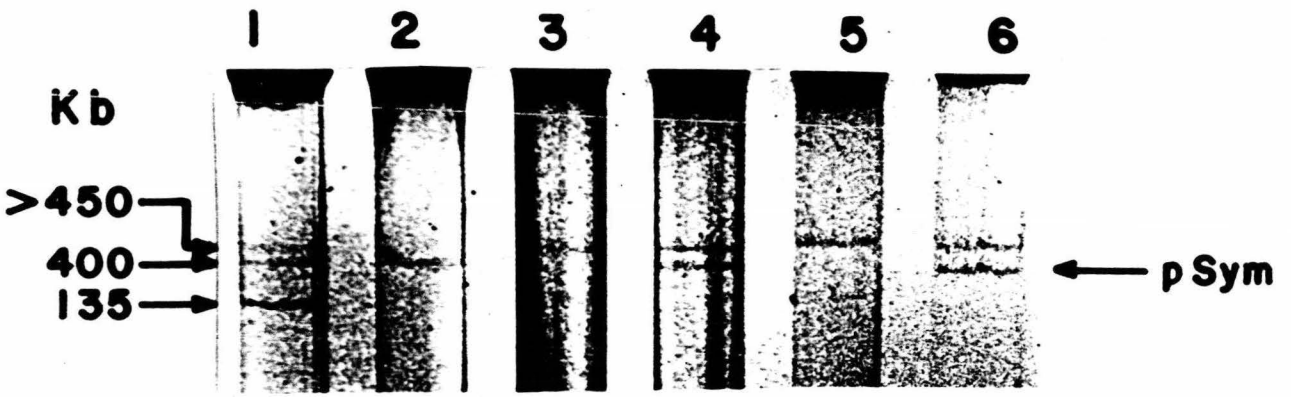


Figure 1

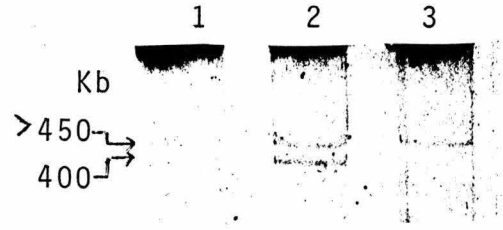
Estabilidad del Fenotipo Simbiótico de las Cepas CFN2515 y CFN4415.

Las cepas CFN2515 y CFN4415 (Tabla 1p 72) fueron tratadas por calor para determinar la frecuencia de deleción del - - plásmido simbiótico de la cepa CFN23 en el fondo genético de -- los "pre-Rhizobium phaseoli" CFN2500 y CFN4400 (Tabla 1p 72); encontré que la resistencia a kanamicina de ambas cepas se pierde con una frecuencia mucho menor que la resistencia a kanamicina de la cepa CFN2315 (Tabla 1p 72), estos resultados se muestran en la siguiente tabla.

Frecuencia de derivados sensibles a Kanamicina.

Cepa	% Kan^S
2315	84
2515	1
4415	2

Los derivados sensibles a kanamicina de la cepa CFN2315 conservan un plásmido simbiótico deletado, pero sin un cambio aparente en el peso molecular (p 30). Se analizó el perfil - de plásmidos de la cepa sensible a kanamicina derivada de la - - CFN4415 y se encontró que esta cepa pierde todo el plásmido simbiótico, estos resultados se muestran en la siguiente figura:



Perfil electroforético de plásmidos. Carriles 1) CFN 4400 2) CFN4415 3) derivado sensible a kanamicina de la cepa CFN4415. Se muestra el peso molecular de los plásmidos expresados en kilobases (Kb).

La diferencia encontrada, tanto en frecuencia de aparición de cepas sensibles a kanamicina al heredar el pSym de la cepa CFN2315 como en el mecanismo de pérdida de ésta resistencia, entre las cepas de Rhizobium phaseoli CFN23 y CFN42 -- (p 40) y los "pre-Rhizobium phaseoli" CFN2500 y CFN4400, pueden ser debidas a que el mecanismo de recombinación de las cepas CFN2500 y CFN4400 sea distinto del de los verdaderos R.phaseoli, o a que el rearreglo génico que da como resultado la inestabilidad de la información simbiótica, requiera la presencia de otros plásmidos.

Todas las cepas de R. phaseoli que pierden el fenotipo simbiótico con alta frecuencia presentan un plásmido de 135Kb (Fig. 1p 26), este plásmido tiene secuencia de DNA homólogas a el plásmido simbiótico (1), se transfiere con alta frecuencia (2) y los genes de la nitrogenasa reductasa (nifH) se encuentran asociados a él en algunas cepas (2), (Fig. 1p 26); por todas -

estas características el plásmido de 135Kb es un posible candidato para participar en el rearreglo génico que da como resulta do la inestabilidad del fenotipo simibiótico de R. phaseoli, -- para descartar esta hipótesis se transferirá el plásmidos de - 135Kb y el simbiótico de la cepa CFN23 a las cepas de pre-Rhizo bium phaseoli CFN2500 y CFN4400 y se determinará la estabilidad de información simbiótica.

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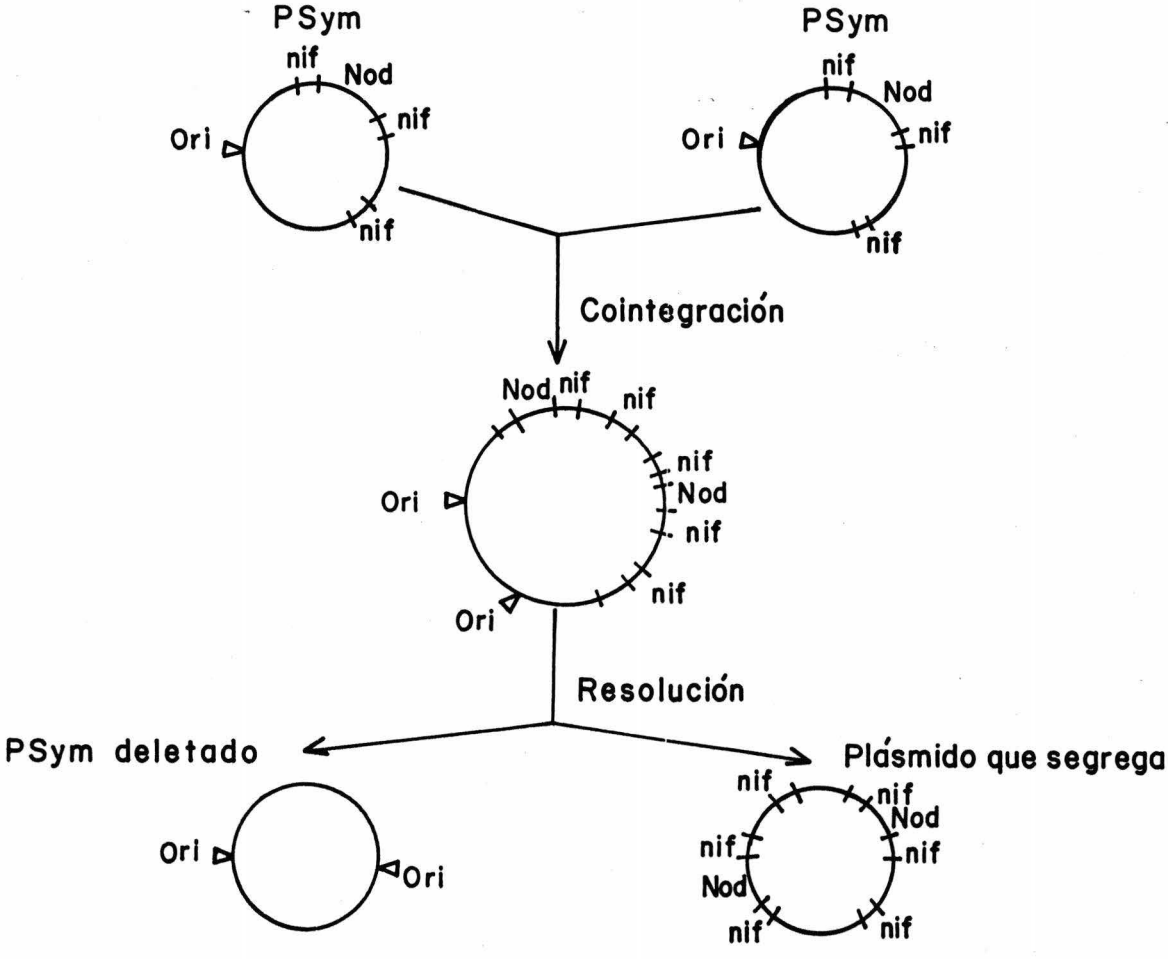
DISCUSIUN

El rearreglo génico que tiene como consecuencia que algunas cepas de R. phaseoli pierdan la capacidad de nodular frijol con una frecuencia elevada es producto de la recombinación del plásmido simbiótico (pSym), el resultado de esta recombinación es la sustitución de una parte del pSym en la que se encuentran los genes estructurales de la nitrogenasa y por lo menos parte de la información necesaria para la nodulación, por otras secuencias de DNA. Esto se ejemplifica en el siguiente modelo:

Modelo de deleción-sustitución del Plásmido Simbiótico de Rhizobium phaseoli CFN23



El modelo presupone la recombinación entre dos replicones que forman un cointegrado y su resolución en la que se intercambian secuencias. Uno de los replicones que intervienen en la recombinación es necesariamente el plásmido simbiótico, y el otro puede ser otra molécula del mismo plásmido (como se presenta en el esquema), u otro plásmido presente en la cepa; una condición del modelo es que la molécula resultante que lleva los genes de la nitrogenasa sea incapaz de replicarse en la bacteria.

Todas las cepas inestables de R. phaseoli contienen un plásmido de 135Kb, (Fig 1, p26), en la cepa CFN23 este plásmido tiene homología estructural con el pSym y los genes de la nitrogenasa se pueden asociar a él (1), en la cepa CFN5 los genes de la nitrogenasa se encuentran tanto en el pSym como en este plásmido. (Flores, M. comunicación personal). Este plásmido pudiera recombinar con el pSym y participar en la inestabilidad del fenotipo simbiótico de R. phaseoli; la presencia de este plásmido no es suficiente para que una cepa sea inestable, pues existen cepas como la CFN42 que tienen esta molécula y pierden su capacidad de nodular frijol con muy baja frecuencia (1)

Para la inestabilidad simbiótica de una cepa es importante también la estructura de su pSym, ya que como se discutió en el artículo "Formation of a Rhizobium phaseoli Symbiotic Plasmid by Genetic Recombinación", existen dos tipos de pSym en R. phaseoli y solo uno de ellos participa en el rearreglo génico que da como resultado la inestabilidad del fenotipo simbiótico.

El plásmido simbiótico de la cepa CFN2315 (Tabla1 p29) participa con mayor frecuencia en el rearreglo de deleción sustitución cuando se encuentra en el fondo genético de una mutante de la cepa CFN23 que tiene aumentada la frecuencia de recombinación homóloga (Soberón-Chávez G. y Nájera R. manuscrito en preparación): esto sugiere que sea la recombinación legítima la involucrada en la generación de deleciones en el pSym de la cepa CFN23. La caracterización de mutantes de la cepa CFN23 con una frecuencia disminuida de perdida de la capacidad simbiótica aclarará la participación de la recombinación homóloga en la deleción-sustitución de su pSym.

El genoma de las Rhizobiaceas presenta gran cantidad de secuencias de DNA reiteradas (Flores M. et al manuscrito mandado a publicación), estas secuencias reiteradas están presentes en el pSym, y pudieran aumentar la frecuencia de recombinación propiciando así los rearreglos génicos; sin embargo la presencia de reiteraciones no es suficiente para que una cepa de R. phaseoli sea inestable; tanto el pSym de la cepa CFN42 como el de la CFN23 presentan secuencias reiteradas (Flores M. comunicación personal) y solo el pSym de la cepa CFN23 pierde los genes estructurales de la nitrogenasa con alta frecuencia. (Tabla2 p 55)

El hecho de que apartir de la pSym deletado de la cepa CFN23 y de un R' derivado del pSym de la cepa CFN42 se pueda generar un plásmido simbiótico "inestable" (Tabla2 p 55), y de que se pueda generar este tipo de pSym inestable a partir de la recombi-

binación del R' y de un plásmido indígena de la cepa CFN42 (Ta- blaz p50 sugiere que el tipo de pSym inestables es un mosaico de dos tipos de secuencias de DNA que pueden o no estar asociados, la disociación de esta información podría compensar la gran frecuencia de pérdida de la información simbiótica. Si una parte - del pSym inestable se transfiere entre bacterias con una frecuencia elevada al asociarse con un plásmido conjugativo (como el plásmido de 135Kb de la cepa CFN23 que se transfiere con una frecuencia de 2×10^{-3}) y el resto de la información no se pierde (el pSym deletado de la cepa CFN23 se pierde con una frecuencia menor a 1×10^{-5} y el plásmido e de la cepa CFN42 es también muy estable), una bacteria que hereda un pSym inestable podría llegar a tener un equilibrio entre la pérdida de parte del plásmido por recombinación y su reintegración al heredarlo por conjugación.

El pSym de la cepa CFN42 no complementa a la cepa de - Agrobacterium tumefaciens GMI9023 para la formación de nódulos "sanos" de frijol , mientras que el pSym de la cepa CFN23 y el que se forma por cointegración del plásmido de la cepa CFN42 y el R' 10 si lo hacen (datos no mostrados). Mutaciones en el plásmido e de la cepa CFN42, confieren un fenotipo Fix^- a la bacteria (2). Todo esto sugiere que toda la información simbiótica plasmidiana, en la cepa CFN23 se encuentra en el pSym, y que en la cepa CFN42 se encuentra por lo menos en el pSym y en el plásmido e. Será interesante determinar si otras cepas estables de "R. phaseoli. tienen el mismo arreglo de la información

simbiótica que la cepa CFN42, o si existe un pSym estable que - contenga toda la información simbiótica.

El estudio de la inestabilidad del fenotipo simbiótico de R. phaseoli desde varios enfoques me ha permitido el conocer y manipular el genoma de esta bacteria hasta llegar a proponer algunos modelos de la dinámica de la información simbiótica de esta bacteria. El vulnerar estos modelos y el evaluar la importancia que tienen dentro del establecimiento y la perpetuación de la simbiosis entre R. phaseoli y el frijol seran los objetivos de mi investigación a mediano plazo.

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