EFICIENCIA DE NODULACION DE *Rhizobium tropici*: PAPEL DE LA CITRATO SINTASA PLASMIDICA Y DE LA PRODUCCION DE ACIDO INDOLACETICO EN SIMBIOSIS.

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Tesis de Doctorado

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CONTENIDO

Introducción.

El nitrógeno es un elemento indispensable para todos los seres vivos. Aunque es muy abundante en la atmósfera terrestre, representando aproximadamente el 78% de ella, su estado químico es inaccesible para la gran mayoría de los seres vivos. Estos requieren de nitrógeno combinado en forma de amonio, nitrato o compuestos orgánicos. Existen, sin embargo, organismos procariotes capaces de transformar al nitrógeno atmosférico en nitrógeno combinado, principalmente en amonio. A este proceso se le denomina "fijación biológica de nitrógeno". La contribución de la fijación biológica de nitrógeno al total del nitrógeno combinado de la Tierra es incierta. Las estimaciones oscilan entre un 50% y un 90% (88), lo que hace pensar que se requieren estudios más profundos y completos que disminuyan al mínimo esta ambigüedad. El resto del nitrógeno combinado se generaría a través de descargas eléctricas, radiación ultravioleta, etc.

Las bacterias capaces de llevar a cabo la fijación biológica de nitrógeno se han dividido en dos grandes grupos. Uno de estos grupos comprende a microorganismos de los géneros *Klebsiella, Anabaena, Azospirillum* y *Acetobacter*, entre otros, los cuales fijan nitrógeno con el fin de cubrir sus propios requerimientos nutricionales; a éstos se les denomina fijadores de nitrógeno en vida libre. El segundo grupo representa, entre otros, a bacterias de los géneros *Rhizobium, Bradyrhizobium* y *Azorhizobium*, los cuales son capaces de establecer una asociación simbiótica con ciertas plantas, principalmente leguminosas, y fijar nitrógeno durante esta asociación; por ello se les denomina fijadores simbióticos de nitrógeno. A este grupo nos referiremos genéricamente como *Rhizobium*, a menos que se requiera especificar. Ambos grupos han generado gran interés debido a su contribución al ciclo del nitrógeno y su estudio seguramente tendrá un fuerte impacto en la ecología y la agronomía.

La interdependencia entre especies es una generalidad en la naturaleza. Esta interdependencia es especialmente evidente en el mundo de la interacción planta-bacteria. De hecho, no existe planta axénica en forma natural. La mayoría de estas interacciones han sido poco estudiadas tanto al

nivel fisiológico como al nivel molecular.

Los microorganismos interactúan con las plantas para nutrirse. Se distinguen tres grupos de bacterias de acuerdo a su forma de interacción: las saprófitas, las patógenas y las simbióticas. Las saprófitas se nutren de los exudados de las raíces y pueden ser benéficas para la planta, como el caso de *Azospirillum* . Las patógenas infectan y enferman a la planta; donde el ejemplo mejor conocido es el de *Agrobacterium tumefaciens* , el cual ha desarrollado una estrategia infectiva especialmente sofisticada (2).

El interés de la presente tesis se centra en el tercer grupo: las simbióticas, en especial a *Rhizobium*. El proceso simbiótico de *Rhizobium* es un excelente modelo de estudio en la biología del desarrollo. Ofrece la posibilidad de manipular a los simbiontes por separado, lo que ha permitido disecar tanto genética como molecularmente al proceso (46,51).

Taxonomía de *Rhizobium*.

El análisis filogenético de *Rhizobium* nos dá un marco de referencia para estudiar las estrategias de interacción simbiótica o patogénica de las *Rhizobiaceae* con las plantas. De ahí nació la necesidad de determinar con la mayor certeza posible la posición taxonómica de las distintas especies de *Rhizobium* , en particular del modelo de estudio de la presente tesis, clasificada inicialmente como *R. leguminosarum* bv. phaseoli tipo II.

La familia de las Rhizobiaceae comprende cinco géneros: *Rhizobium, Bradyrhizobium, Agrobacterium, Phyllobacterium* y *Azorhizobium*.

Rhizobium y *Bradyrhizobium* forman nódulos fijadores de nitrógeno en las raíces de leguminosas; *Azorhizobium* forma nódulos en el tallo de*Sesbania*. *Phyllobacterium* produce hipertrofias en las hojas de ciertas plantas y *Agrobacterium*, a excepción de *A. radiobacter*, produce tumores en tallos y raíces de plantas dicotiledóneas.

A pesar de que *Rhizobium* y *Bradyrhizobium* comparten estrategias similares de infección y poseen genes similares de nodulación y fijación de nitrógeno, cromosomalmente están muy distantes entre sí (41). Tradicionalmente estas especies se clasificaron de acuerdo a su posibilidad de nodular a un hospedero específico. Sin embargo, pronto se hizo evidente lo limitado de este parámetro taxonómico, resaltando la promiscuidad infectiva de estas bacterias por diferentes plantas. Por ejemplo, *Phaseolus vulgaris* (frijol) es nodulado por *R. tropici* (ver adelante) y *R. etli* (52) y por otros rhizobia.

Ha sido necesario recurrir a otros análisis que nos ubiquen con mayor precisión la posición taxonómica de una determinada especie. Para ello se han desarrollado técnicas que comprenden comparaciones de DNA-DNA, electroferotipos y últimamente, el análisis de secuencias de nucleótidos , entre lo que destaca el análisis comparativo de secuencias de genes de RNA ribosomal.

Los rhizobia capaces de nodular frijol clasificados hasta 1983 como *R. phaseoli*, posteriormente fueron reclasificados como *R. leguminosarum* biovar phaseoli (43). Los otros dos biovares de la especie, viciae y trifolii, nodulan chícharo y trébol, respectivamente. Sin embargo, en el biovar phaseoli se detectó una gran heterogeneidad al utilizar los diferentes criterios mencionados antes. Comparando secuencias del plásmido simbiótico se encontraron dos grupos: el tipo uno contiene reiterados los genes de la nitrogenasa, sólo puede nodular frijol y contiene al gene *psi*, involucrado en la inhibición de la síntesis de polisacáridos (8). El tipo dos no tiene reiterados los genes de la nitrogenasa, no contiene *psi* y presenta un amplio rango de infección (52). Presenta además otras características distintas al tipo uno, como que es tolerante a la acidez y a altas concentraciones de aluminio, sus polisacáridos extracelulares son químicamente distintos y su plásmido simbiótico permite nodular y fijar nitrógeno a *Agrobacterium* (53). Ante estas evidencias, decidimos definir la posición taxonómica de los tipo dos, al cual pertenece nuestro modelo de estudio. Para ello se realizó el siguiente trabajo*:

* Si se desea consultar el artículo completo, véase el anexo.

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Rhizobium tropici, a Novel Species Nodulating *Phaseolus vulgaris* L. Beans and *Leucaena* sp. Trees

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A new *Rhizobium* species that nodulates *Phaseolus vulgaris* L. and *Leucaena* spp. is proposed on the basis of the results of multilocus enzyme electrophoresis, DNA-DNA hybridization, an analysis of ribosomal DNA organization, a sequence analysis of 16S rDNA, and an analysis of phenotypic characteristics. This taxon, *Rhizobium tropici* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli (type II strains) and was recognized by its host range (which includes *Leucaena* spp.) and *nif* gene organization. In contrast to *R. leguminosarum* biovar phaseoli, *R. tropici* strains tolerate high temperatures and high levels of acidity in culture and are symbiotically more stable. We identified two subgroups within *R. tropici* and describe them in this paper.

Members of the genus *Rhizobium* nodulate the roots of leguminous plants. The rhizobia that infect peas, clovers, and beans (*Phaseolus vulgaris* L.) are clustered in a single species, *Rhizobium leguminosarum* (29), which has three biovars (*Rhizobium leguminosarum* biovar viciae, *Rhizobium leguminosarum* biovar trifolii, and *Rhizobium leguminosarum* biovar phaseoli); these biovars contain different symbiotic plasmids that encode distinct nodulation specificities. Nevertheless, heterogeneity in *Rhizobium leguminosarum* biovar phaseoli has been identified by using such different criteria as protein pattern (50), antibiotic resistance (2), serological type (49), multilocus enzyme electrophoresis behavior (45), DNA-DNA hybridization data (10, 26, 54), plasmid profile (37), and exopolysaccharide structure (70).

We previously distinguished two different types among isolates obtained from bean nodules and found differences in their symbiotic plasmids (36, 38, 39). Type I strains have multiple copies of nitrogenase *nifH* genes (39, 46), a narrow nodulation host range, and hybridize with the *psi* (polysaccharide inhibition) gene (3). Type II strains have single copies of *nif* genes, nodulate *Leucaena* spp., and do not hybridize with the *psi* gene (36, 39).

Type II strains have received attention because their symbiotic plasmids promote an effective and completely differentiated symbiotic process in *Agrobacterium tumefaciens* recipients (5, 38). They are genetically stable, retaining their symbiotic plasmid after prolonged incubation at 37°C. Some are heat tolerant (31) or acid and aluminum resistant (12, 25, 30, 62). The nodulation genes from one of these strains have been cloned (64). The chemical composition and structure of the extracellular polysaccharides from one type II strain differ from the chemical composition and structure of the extracellular polysaccharides from type I isolates (23).

Type II strains have been less successful in competition for bean nodule occupancy than the type I strains used (41). The former have been reported to occur less frequently in bean nodules (39). Nodule occupancy by type II strains can be enhanced under acid conditions (47, 63).

To define the taxonomic position and the genetic relatedness of type II strains, we analyzed 64 type II strains having different geographical origins and compared them with other species of rhizobia.

For a long time multilocus enzyme electrophoresis has been a standard method used in systematics (44), and this method is perhaps the best approach in large-scale studies to estimate the genetic diversity and structure of related populations (55, 67, 68). The results of multilocus enzyme electrophoresis studies provided the basis for the identification of two previously undescribed species among *Legionella pneumophila* strains (57) and identified two groups of bacteria within *Rhizobium meliloti* (19). Our strategy was to order type II strains by multilocus enzyme electrophoresis and then to characterize these bacteria phenotypically. Representative strains were chosen for total DNA and ribosomal DNA hybridization and for the determination of partial 16S rRNA gene sequences.

On the basis of the criteria analyzed, we propose a new species, *Rhizobium tropici*, which contains two subgroups that correspond to type IIA and type IIB strains.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1.

Growth conditions. Rhizobia were maintained on yeast extract-mannitol (YM) medium (65), on peptone-yeast extract (PY) medium, (43), or in minimal medium (MM) (17) containing different substrates. Average doubling times were estimated from optical densities recorded at 600 nm every 2 h in PY medium at 30°C. Bacterial swarming was tested by growing strains for 2 days on PY medium supplemented with 0.3% agar.

Nodulation and nitrogen fixation were tested in sterilized Leonard jars (65) containing vermiculite and sand by using *P. vulgaris* cv. Carioca 80 and *L. leucocephala*.

Multilocus enzyme electrophoresis. Cultures derived from single colonies were grown overnight at 30°C in 50 ml of PY

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En este trabajo describimos a una nueva especie de *Rhizobium*, *R. tropici* , subdividida en dos tipos A y B. Esta especie difiere en varios aspectos de la especie en la cual estaba contenida anteriormente, esto es, *R. leguminosarum* biovar phaseoli, ahora reclasificada en nuestro laboratorio como *R. etli* (79). Como características fenotípicas reelevantes, *R. tropici* crece en medios ácidos y en altas concentraciones de aluminio, a temperaturas hasta 40 °C y tiene un amplio rango de infección. A nivel molecular se distingue de otras especies por los resultados de hibridización DNA-DNA, por análisis de electroferotipos de enzimas metabólicas y por sus secuencias nucleotídicas de genes ribosomales.

Este análisis nos permite ahora ubicar a nuestro modelo en un contexto diferente al de *R. leguminosarum* y al de *R. etli* en su relación con la planta. Actualmente *R. tropici* ha sido aceptada como una nueva especie por el Comité Internacional de Taxonomía, Subcomité *Agrobacterium* y *Rhizobium*. En la tabla 1 se muestra cómo se han clasificado a los rhizobia hasta este momento.

Tabla 1

Especies de Rhizobia

ESPECIE	HOSPEDERO
Rhizobium meliloti	*Medicago*
Rhizobium leguminosarum	
biovar *viciae*	*Pisum, Vicia.*
biovar *trifolii*	*Trifolium*
biovar *phaseoli*	*Phaseolus*
Rhizobium loti	*Lotus*
Rhizobium fredii	*Glycine*
Rhizobium galegae	*Galegae officinalis*
Rhizobium huakuii	*Astragalus sinicus*
Rhizobium xinjiangensis	*Glycine*
Rhizobium tropici	*Phaseolus, Leucaena.*
Rhizobium etli	*Phaseolus*

Asimismo, y por los mismos motivos, quisimos determinar la relación filogenética de aislamientos de rhizobia provenientes de otras leguminosas originarias de Mesoamérica, que nodulan y fijan nitrógeno en *Phaseolus vulgaris*. El resultado de estos estudios se describe en el siguiente trabajo* (enviado a publicación):

* Si desea usted consultar el trabajo completo, véase el anexo.

Rhizobium etli sp. nov. is a branch of American rhizobia with different specificities.

Running Title: Rhizobia related to *R. etli* sp. nov.

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ABSTRACT

The phylogenetic analysis of American rhizobia from tropical legumes was performed by determing the nucleotide sequences of partial fragments of 16S rRNA genes. By these criteria, *R.* spp. are relatives to *R. etli*. *R.* spp. are broad-host range bacteria and *R. etli* sp. nov. is probably the species that coevolved with *Phaseolus vulgaris* bean. *R.* spp. differed from *R. etli* in many plasmid-borne traits. Nod-metabolites produced by *R.* spp. were analyzed. They were different from those produced by *R. etli*, as they were sulfated-labeled oligosaccharides. The study of clusters of genetically related bacteria may help to understand the evolution of specificity in *Rhizobium* spp.

En este trabajo se define a un grupo de rhizobia genéticamente relacionadas entre sí y muy emparentadas con *R. etli* , lo que sugiere un origen común. Estos *Rhizobium spp* provienen del mismo sistema ecogeográfico de *R. etli* pero, a diferencia de éste último, son capaces de establecer una simbiosis con otras leguminosas además del frijol. Sin embargo, *R. etli* supera fuertemente a los *R. spp* en su capacidad de infectar frijol (54). De estos resultados se extrae, y se apoya, la idea de que *R. etli* es el microsimbionte especializado para *P. vulgaris*. No sorprende entonces que en Mesoamérica la gran mayoría de los aislamientos de *Rhizobium* obtenidos de frijol pertenezcan a *R. etli* (52).

Por otra parte, los resultados apoyan la hipótesis interesante acerca de una coevolución entre el micro y el macrosimbionte, puesto que el frijol y la *Leucaena* comparten la región ecogeográfica Además, nuevamente se establece un criterio que separa y ubica a *R. tropici* de otros rhizobia capaces de establecer una relación simbiótica con *Phaseolus vulgaris* .

El Proceso Simbiótico entre *Rhizobium* y la planta.

Establecida la posición taxonómica de *R. tropici*, describo ahora un panorama general del conocimiento actual acerca del proceso simbiótico entre *Rhizobium* y la planta. Mientras que la mayoría de los microorganismos interactúan con las plantas en la superficie o a través de una herida. *Rhizobium* ha desarrollado una estrategia muy especializada que culmina en la endocitosis de la bacteria por la planta en cuyo estado la bacteria es capaz de fijar nitrógeno. Este microbio en su estado simbiótico se comporta como un organelo dado que algunas de sus funciones son fisiológicamente dependientes de su hospedero.

La infección por *Rhizobium* provoca el crecimiento de una estructura planta-bacteria altamente diferenciada llamada nódulo en el cual las bacterias invasoras fijan nitrógeno atmosférico en una forma química tal que las plantas puedan asimilarlo y usarlo para la síntesis de biomoléculas. Por su parte, la planta provee de nutrientes a la bacteria para su manutención. Ya que esta asociación otorga un beneficio mutuo para el invasor (*Rhizobium*) y el invadido (la planta), se le ha llamado simbiosis. Sin embargo, si por algún defecto tanto de la bacteria o de la planta no se logra fijar nitrógeno, entonces la planta infectada puede verse fuertemente

debilitada semejando un estado enfermizo. Por ello, la relación simbiótica entre *Rhizobium* y la planta podría considerarse como una "enfermedad controlada" y a la bacteria como un "patógeno atenuado" (96).

El desarrollo del nódulo en leguminosas templadas ha sido el más estudiado. En este sistema, la interacción planta-*Rhizobium* se inicia por una atracción quimiotáctica de la bacteria por exudados radiculares (31,23, 44). La bacteria coloniza y se adhiere a la punta del pelo radicular. Por parte de la bacteria, esta adherencia es dependiente de exopolisacáridos (EPS) (35), lipopolisacáridos (LPS) (89) y probablemente de ciertas proteínas, como es el caso de la rhicadhesina (distribuída a lo largo de las Rhizobiaceae) (85). En la planta, las lectinas parecen jugar un papel importante en la adherencia bacteriana (45). Aquí podría estar mediado el primer grado de especificidad entre el *Rhizobium* y la planta. Sigue después un enroscamiento del pelo que engloba a la bacteria. Se forma entonces una estructura tubular de origen vegetal denominada hilo de infección que penetra a través de las células externas de la corteza y se ramifica en la corteza. Células internas de la corteza próximas y frontales al hilo comienzan a dividirse formando un foco infectivo. Este meristemo es invadido por las bacterias que se han desplazado a través del hilo de infección. Al invadir la célula vegetal, la bacteria es recubierta por una membrana vegetal llamada membrana peribacteroidal. Las células invadidas detienen su crecimiento no así las células adyacentes que forman nueva corteza y haces vasculares hasta que el nódulo está morfológicamente definido. Las bacterias envueltas en esta membrana pronto se diferencian en bacteroides que es el estado en que son capaces de fijar nitrógeno.

Existe otra forma de infección por penetración intercelular. El lugar de penetración de la bacteria bien podría ser el lugar de emergencia de una raíz secundaria o una herida eventual. Este modo de infección está descrito para *Neptunia oleracea* (7), *Arachis* sp. (cacahuate) (15) y *Mimosa scabrella* (17), entre otras. Generalmente un *Rhizobium* determinado no posee el potencial de infectar de varias maneras, aunque existen excepciones (57). Una revisión más extensa acerca del modo de infección se encuentra en Rolfe y Gresshoff (1988) (72).

Durante el desarrollo del nódulo varios genes específicos tanto de la bacteria como de la planta se expresan coordinadamente en el tiempo y el espacio. Por parte de la planta se han encontrado un gran número de proteínas que aparecen sólo en nódulo (nodulinas). Un ejemplo de nodulina es la leghemoglobina. Esta proteína, en combinación con un grupo hemo sintetizado por la bacteria, se sintetiza sólo en nódulo y al tiempo de la

fijación de nitrógeno.

Las bacterias simbióticas fijadoras de nitrógeno *Rhizobium*, *Bradyrhizobium* y *Azorhizobium* poseen la información genética necesaria para dirigir la formación del nódulo. En el *Rhizobium*, se han aislado distintas clases de mutantes afectadas en un determinado punto del proceso simbiótico. El aislamiento y caracterización de distintos genes simbióticos identificados generalmente a través de mutaciones ha permitido obtener un panorama cada vez más definido de las funciones bacterianas necesarias en el establecimiento de una simbiosis exitosa.

En *Rhizobium* se ha encontrado que la gran mayoría de las mutaciones generadas que alteran de alguna manera el proceso simbiótico, se ubican en plásmidos de alto peso molecular a los que se les ha llamado plásmidos "simbióticos" (pSim) (62,34,74) En *Bradyrhizobium* y *Azorhizobium* estos mismos genes se localizan en el cromosoma (61,97).

Los genes de nodulación se expresan en presencia de diferentes compuestos exudados por la raíz de la planta (39,58). Estos compuestos se han identificado como moléculas fenólicas de bajo peso molecular (26,65, 70,100) que derivan de la ruta de los fenilpropanoides de la planta implicados en la respuesta de defensa (72). Se ha encontrado que un mismo exudado de raíz puede contener varios de estos compuestos, comunmente llamados flavonoides . Se han detectado también flavonoides que actúan como anti-inductores (18).

El proceso inductivo por flavonoides se lleva a cabo mediante la activación del producto del gene regulatorio *nod*D (58,37,75,83) La evidencia sugiere que el flavonoide se une al producto de *nod*D, cambiando su conformación y tal vez convirtiéndolo en una forma soluble ya no unida a la membrana, capaz así de unirse a un pequeño fragmento de DNA regulatorio llamado "nod-box" de aproximadamente 50 pb, que se encuentra antes del inicio de transcripción de varios genes de nodulación (47,76,81). *nod*D se encuentra en multicopia en varias especies de *Rhizobium* (32,36,33,3,98) y sus productos poseen distintas afinidades por diferentes flavonoides, lo que representa otro grado de especificidad de la bacteria por la planta (86,33). Algunas mutaciones en el gene *nod*D pueden alterar su habilidad para activar los genes *nod*, cambiando su afinidad por activadores (modificando por ende su espectro de infección) o eliminando su dependencia de activarse por flavonoides (19, 86).

De los genes de nodulación activados por el producto de *nod*D se encuentran los genes *nod*ABC, llamados genes de nodulación "comunes" debido a que estos mismos genes en otras especies se encuentran muy conservados y porque mutaciones en ellos pueden ser complementadas por

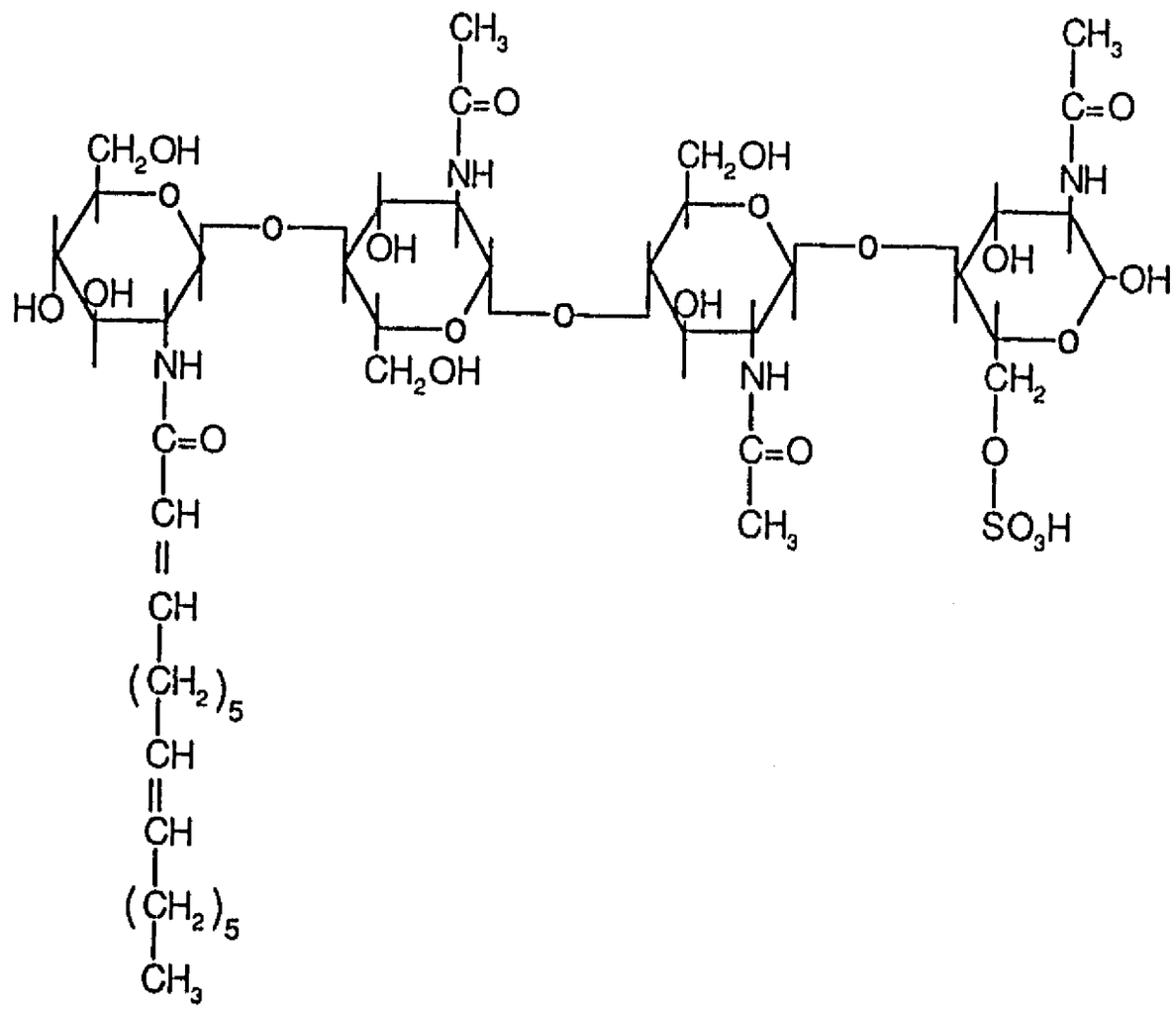
sus homólogos de otras especies (20,78). Los productos de los genes *nod* comunes son necesarios para la deformación del pelo radicular y para inducir la proliferación celular del foco infectivo en el inicio de la interacción simbiótica (95,24). Los genes de nodulación estan involucrados en la biosíntesis de un lipo-oligosacárido llamado "factor de nodulación" que es quien induce la deformación del pelo absorbente y la actividad meristemática del foco infectivo (48) (fig.1). El producto de *nod*C posee características de una proteína membranal y se le ha encontrado homología estructural con genes de quitino sintetasa (42,9,87), lo que sugiere que *nod*C está involucrado en la biosíntesis del esqueleto de azúcar del factor de nodulación. Por su parte, *nod*A y *nod*B parecen estar involucrados en la acetilación y acilación de este factor (87) Cualquier mutación que elimine a los productos o algún producto de los genes *nod*ABC genera un fenotipo Nod- (22,55).

Otros genes de nodulación también regulados por compuestos exudados de la planta son los *hsn* (host specific nod genes) (39,83). Mutaciones en estos genes no son complementados por sus homólogos de otras especies (38;16) y generalmente alteran el espectro de infección de la bacteria (21,59). Se ha encontrado que los *hsn* modifican químicamente al lipo-oligosacárido básico sintetizado por los productos de los *nod* comunes, estableciendo de esta manera otro nivel de especificidad.(82,49) (fig.1).

Una vez que la bacteria se interna a las células de la corteza (ya sea por hilo de infección o por penetración intercelular), ésta se desplaza por crecimiento hacia las células corticales internas donde infectará algunas de ellas. En este lapso dejan de expresarse los genes tempranos de nodulación y dan paso a la expresión de otros , que comprenden a los genes de diferenciación a bacteroide, los genes estructurales de la nitrogenasa, los genes involucrados en la biosíntesis de cofactores necesarios para la fijación de nitrógeno, entre otros. En *Klebsiella pneumoniae* , bacteria fijadora de nitrógeno en vida libre, son más de veinte genes arreglados en diferentes operones los requeridos para fijar nitrógeno (11,4). Algunos de los genes de *Rhizobium* o *Bradyrhizobium* son equivalentes tanto estructural como funcionalmente a algunos de los genes de fijación de nitrógeno (genes *nif*) de *K. pneumoniae*. Estos incluyen al operón *nif*HDK que codifican para los genes estructurales de la nitrogenasa (77,13,68), al gene regulatorio *nif*A (27,90,1) y otros (25).

Una característica interesante encontrada inicialmente en *R. etli* y después en otras especies como *R. fredii* y *A. caulinodans* es la de poseer reiterados los genes estructurales de la nitrogenasa (68,60,67). En *R. etli*

Fig. 1



NodRmI

Factor de Nodulación de *Rhizobium meliloti*

existen dos operones *nif*HDK y una copia extra de *nif*H (69). Ambos operones son funcionales dado que al mutar cualquiera de los dos la fijación de nitrógeno disminuye (73).

Antecedentes particulares al primer trabajo.

Entre los genes bacterianos identificados que inciden sobre el proceso simbiótico se encuentran los *nod* comunes, los *hsn* , los *exo*, los *nif* y los *fix*. La mayoría de ellos afectan de una manera u otra el desarrollo del nódulo *per se*. Sin embargo, algunos de ellos inciden sobre funciones que no estan involucradas directamente en la formación del nódulo, como por ejemplo los genes *exo* B, G y J de *B. japonicum*, cuya ausencia provoca una disminución en la competividad por nodular (63,101). Estas funciones, aunque no esenciales para la morfogénesis del nódulo, pueden ser determinantes para que una bacteria logre infectar a una planta. En experimentos de inoculación a suelos con *Rhizobium* se ha encontrado que difícilmente compiten con los autóctonos (92). Se consideró entonces que el estudio de genes involucrados en procesos de competencia y/o eficiencia de nodulación nos permitiría conocer qué funciones son importantes para la bacteria que le harán nodular exitosamente en un medio ambiente sumamente competitivo

De una mutagénesis al pSim de *R. tropici* cepa CFN299 con el transposón Tn5-*mob* se generaron un conjunto de mutantes con un fenotipo simbiótico alterado en *P. vulgaris*. Entre ellas se eligió a una (mutante CFNE130) que presentaba el siguiente fenotipo simbiótico: los nódulos que induce son indistinguibles de los de la cepa silvestre, no tiene retraso de nodulación ni cambia su especificidad por el hospedero. Su defecto consiste en que sólo es capaz de inducir la mitad del número de nódulos con respecto a la silvestre, esto es, tiene problemas con respecto a su eficiencia de nodulación

El estudio de esta mutante y del gene o genes interrumpidos por el transposón nos podría esclarecer alguna función del *Rhizobium* importante para elevar el número de simbiosis con la planta. El presente trabajo conlleva estudios moleculares, genéticos y fisiológicos de la mutante CFNE130.

Nodulating ability of *Rhizobium tropici* is conditioned by a plasmid- encoded citrate synthase.

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SUMMARY

Rhizobium species elicit the formation of nitrogen-fixing root nodules through a complex interaction between bacteria and plants. Various bacterial genes involved in the nodulation and nitrogen fixation processes have been described and most have been localized on the symbiotic plasmids (pSym). We have found a gene encoding citrate synthase on the pSym plasmid of *Rhizobium tropici*, a species which forms nitrogen fixing nodules on the roots of beans (*Phaseolus vulgaris)* and *Leucaena spp* trees. Citrate synthase is a key metabolic enzyme that incorporates carbon into the tricarboxylic acid cycle by catalyzing the condensation of acetyl-CoA and oxaloacetic acid to form citrate. *Rhizobium tropici pcs*A (plasmid citrate synthase) is closely related to the corresponding genes of Proteobacteria. *pcs*A inactivation by a Tn5-*mob* insertion causes the bacteria to form fewer nodules (30-50% of the original strain) and to have a decreased citrate synthase activity in minimal medium with sucrose. A clone carrying the *pcs*A gene complemented all the phenotypic alterations of the *pcs*A- mutant, and conferred *R. leguminosarum* bv. *phaseoli* (which naturally lacks a plasmid citrate synthase gene) a higher nodulation and growth capacity in correlation with a higher citrate synthase activity. We have also found that *pcs*A gene expression is sensitive to iron availability, suggesting a possible role of *pcs*A on iron uptake.

The discovery of the tricarboxylic acid (TCA) cycle and its central role in carbon metabolism was one of the most significant events in the development of modern biochemistry. Citrate synthase is considered as the rate limiting step of the cycle. With a few exceptions, the TCA cycle has been found in the majority of organisms (Weitzman and Danson, 1976).

Up to now genes encoding central metabolic pathway enzymes such as TCA genes have been located on chromosomes in bacteria, while genes considered accessory or dispensable have been located in plasmids. In *Rhizobium*, genes involved in plant interaction have been located in the so-called symbiotic plasmids (pSym). These carry nodulation and nitrogen fixation genes that determine the formation of symbiotically effective nodules on the roots of specific legumes. We have described a new species of *Rhizobium* (*Rhizobium tropici*) that effectively nodulates *P. vulgaris* and *Leucaena* spp., and tolerant to high temperatures and acidity (Martínez-Romero et al., 1991). In this paper we report the sequence of a functional citrate synthase encoded by the *R. tropici* symbiotic plasmid and some features of its transcriptional regulation and its role in nodulation.

Results and Discussion

Rhizobium tropici CFN299 was mutagenized with Tn5-*mob* (Simon, 1984), and Tn5-*mob* insertions located on the 400 Kb symbiotic plasmid were selected as follows. Five thousand mutants were individually mated (Martínez et al., 1987) with a plasmid-free *Agrobacterium tumefaciens* strain GMI9023 (Rosenberg and Hughet, 1984). *Agrobacterium* transconjugants bearing the Sym plasmid were identified by positive hybridization to the *R. phaseoli nif*H gene (Quinto et al., 1985). The corresponding parental *Rhizobium* strains (300 mutants), with Tn5-*mob* insertions on pSym, were selected and tested for nodulation kinetics and acetylene reduction activity in duplicated assays with *Phaseolus vulgaris* bean plants (Martínez et al.,1985; Martínez-Romero and Rosenblueth, 1990). A mutant (CFNE130) with a deficient nodulation phenotype but with normal nitrogen-fixing capacity was chosen for further analysis. Its Tn5-*mob* single insertion and its flanking regions were cloned in pUC18. The DNA sequence revealed that the Tn5-*mob* was inserted in an ORF that resembles prokaryotic citrate synthase genes (Fig.1). To verify further the plasmid location of the citrate synthase gene, two experiments were performed: 1) A *pcs*A internal fragment (Fig.1a) was used as a probe for hybridization against a CFN299 plasmid profile. A positive hybridization was found in the pSym (Fig. 2a). Southern blot hybridization of strains CFN299, CFNE130 and CFNE299-10 (a spontaneous symbiotic plasmid deletion mutant) showed that CFN299 has two hybridization bands, one corresponding to the plasmid-located (6 Kb) and the other to the chromosomal gene (9 Kb).

CFNE299-10 showed only the 9 Kb band, while CFNE130 has the expected altered hybridization pattern due to the Tn5-*mob* insertion (Fig. 2b). 2) A symbiotic plasmid with a Tn5 derivative, Tn5233 (De Vos et al., 1986), located other than in *pcs*A gene, was transferred to a CFNE130 rifampicin resistant strain (CFNE131). The transconjugant strain CFNE132 loses the original *pcs*A mutant plasmid as revealed by hybridization with a *pcs*A internal fragment (not shown). These data show unambigously a symbiotic plasmid location of the *pcs*A gene.

The *pcs*A deduced amino acid sequence showed 66%, 67%, 62% and 67% homology identity with the products of *E. coli* (Ner et al., 1983), *Pseudomonas aeruginosa* (Donald et al., 1989), *Rickettsia prowazekii* (Wood et al., 1987) and *Acetobacter aceti* (Fukaya et al.,1990) *glt*A respectively. The cladogram that considers the genetic distance between several citrate synthase genes clearly locates *pcs*A gene among those from Gram-negative bacteria (Fig. 3). The conserved amino acids of citrate synthase constituting the active site involved in substrate binding (Bhayana and Duckworth, 1984; Weigand et al., 1984) are also found in the *pcs*A deduced protein (Fig. 1b). In *E. coli*, *sdh*CDAB and *suc*ABCD which code for TCA cycle enzymes, are located upstream of *glt*A (Nimmo, 1987). The 2.5 Kb sequence preceding *pcs*A does not have any homology to other TCA cycle genes (not shown).

CFNE130 grows less and has around 40% of the wild type citrate synthase activity when grown in MM with sucrose as a carbon source (Fig. 4). Sucrose is the main carbon compound in *P. vulgaris* bean phloem (Fisher, 1978) and may be an important nutrient for the bacteria in the infection thread. The reduced nodulation capacity (Fig. 5) may be related

to growth deficiencies of CFNE130 during the infection process. No growth-rate differences or decreased citrate synthase activity were observed when bacteria were grown in a complete medium (not shown).

The wild *pcs*A gene and its flanking regions were cloned in the broad host range pRK7813 plasmid (see Experimental Procedures) and introduced to CFNE130 mutant. This clone restored the phenotypic alterations of the mutant strain., namely, nodulation ability (Fig. 5) and optimal growth on sucrose (Fig. 4a). It also restored wild-type citrate synthase activity (Fig. 4b). It seems that *pcs*A gene confers on the bacteria a superior nodulation ability. This is supported by the fact that *R. leguminosarum* bv. *phaseoli* harboring the *R. tropici pcs*A gene (Fig. 5, strain CFNE138) nodulates about 40% more and grows faster than the wild type strain CFN42.

Gene expression regulated by iron was measured in CFN299 by the ß-glucuronidase activity of a pBJ101.3 plasmid (Jefferson et al., 1987) construction (which is stable in *Rhizobium*), carrying the Pst1-BamH1 fragment of *pcs*A (Fig. 1a, see Experimental Procedures). ß-glucuronidase activity increases with the amount of the iron chelator 2-2' dipyridyl present in the medium (Fig. 6). In the wild type strain, citrate synthase activity increases concomitantly with gene expression but not in the mutant strain CFNE130 (Fig. 7). Iron availability is a limiting factor for the growth of many microorganisms (Neilands, 1987) and in *Rhizobium*, iron acquisition is essential for nitrogen fixation. In iron-limited environments bacteria produce siderophores to acquire iron. Citrate functions as an iron chelator in some *Bradyrhizobium japonicum* strains (Guerinot et al., 1990), in *E. coli* (Hussein et al., 1981), and in

other microorganisms (Cox, 1980; Messenger and Ratledge, 1982; Ecker and Emery, 1983). It may also have the same role in *R. tropici*, as this bacteria is naturally found in acidic environments, where iron is not found in the hidroxylated forms. Citrate, which is considered as a low iron-affinity siderophore may well serve as a chelator under these conditions.

No complementation of the nodulation deficiency of CFNE130 was restored by adding either ferric citrate or sodium citrate to the plant nodulation assays. Perhaps citrate is not reaching the bacteria in these assays during the nodulation process.

DNA duplication has long been recognized as an important factor in the evolution of new genes and genome size. Additional copies of chromosomal genes have been located in plasmids of *Rhizobium*. For example, in *R. meliloti nod*P and *nod*Q are homologous to the *E. coli cys*D and *cys*N genes, whose gene products have ATP sulphurylase activity. *nod*P and *nod*Q are involved in the sulphation of the *R. meliloti* nodulation factor (Schwedock and Long, 1990). In *R. leguminosarum* bv. *viciae*, *nod*M is homologous to the *E. coli* housekeeping gene *glm*S, which codes for glucosamine synthase; both genes, *nod*M and *glm*S, have interchangeable functions (Marie et al., 1992).

The biochemical functions of many plasmid-borne genes are largely unknown and this is also true for *Rhizobium* symbiotic plasmids. It is striking that a key metabolic enzyme such as citrate synthase is encoded in a symbiotic plasmid. We would expect a coordinated expression of the plasmidic and chromosomal citrate synthase genes to avoid a bacterial metabolism collapse due to an incapacity of the cell to control its carbon and energy flux. Our results show that the *Rhizobium* symbiosis should

not be considered as an isolated bacterial process but rather as a whole bacterial metabolic adjustment to the host.

Addendum.

Interestingly, calcium limitation also stimulates *pcs*A-gene expression as well as citrate synthase activity. This was not the case for other divalent cations. This data suggests that *R. tropici* may use citrate as a chelator to obtain iron and calcium, which are two important elements for the symbiotic process.

Experimental Procedures

Media and growth conditions

Minimal media (MM) was according to Zaat et al. (1987), with 0.2% (w/v) of sucrose used as the carbon source. PY medium (complete medium) contained 0.5% peptone, 0.3% yeast extract and 7 mM of $CaCl_2$. Rhizobia were grown at 30 °C.

Sequencing strategy

The Tn5-*mob* single insertion of strain CFNE130 was cloned in the EcoR1 site of pUC18 plasmid and subclones were constructed in M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). Sequence was determined on both strands using the chain termination method (Sanger et al., 1977) from overlapping clones shown in Fig. 1.

Plasmid profiles

Obtained by the Eckhardt procedure (Eckhardt, 1978).

Enzyme assays

1) ß-glucuronidase assay. Cells were sonicated in GUS extraction buffer

(Jefferson, 1987) that contains 1mM 4-Methyl umbelliferyl ß-D-glucuronide (MUG). 4-Methyl umbelliferone (MU), the product of ß-glucuronidase activity, was measured spectrofluorometrically with excitation at 365 nm, emission at 455 nm.

2) Citrate synthase assay. Cells were disrupted by sonication in Tris-HCl 50 mM pH8. Activity was measured spectrophotometrically at 412 nm by DTNB [5-5'-dithiobis(2-nitrobenzoate)] reduction according to Halper (Halper and Srere, 1977).

Hybridization conditions

Probes were labeled with ^{32}P by nick-translation (Rigby et al., 1977). DNA was transferred from agarose gels to nitrocellulose filters as described by Southern (Southern, 1975).

Assay for nodulation and nitrogen fixation

Nodulation assays were performed in agar flasks and in vermiculite jars using *P. vulgaris* Negro Jamapa as described (Martínez et al., 1985; Martínez-Romero and Rosenblueth, 1990). Nitrogenase activity was measured by acetylene reduction.

Plasmid constructions

1) pMP6 was constructed by ligating the Pst1-BamH1 fragment (Fig. 1a) into the Pst1-BamH1 sites of the polylinker of pBJ101.3 plasmid

(Jefferson et al., 1987)., which generates a translational fusion with GUS. 2) Using the PCR procedure, the 2 Kb Pst1-Xba1 fragment containing the entire *pcs*A gene, 0.5 Kb upstream and 0.2 Kb downstream of *pcs*A (Fig. 1), was amplified and cloned in pRK7813 plasmid (Stanley et al., 1987), generating plasmid pMP7. Since pRK7813 can be maintained both in *E. coli* and *Rhizobium*, pMP7 was transferred by conjugation to the *pcs*A-*R. tropici* CFNE130 mutant (generating strain CFNE137) and to *R. leguminosarum* bv. *phaseoli* CFN42 (generating strain CFNE138).

Determination of genetic distances, sequence and phylogenetic analysis

Sequence were aligned using the program Pile Up from the Genetics Computer Group Sequence Analysis Package (Devereux, 1984). The Kimura corrected distance was calculated for each pair of aligned sequences. Trees were obtained with the Fitch Margoliash and Least Squares Methods with an evolutionary clock using the program Kitoch87 from J. Felsentein's PHYLIP 3.4 package (Fitch and Margoliash, 1967).

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Fig. 1 a, Physical map of the *pcs*A locus showing the GUS and Tn5-*mob* insertions. b, Complete nucleotide sequence and deduced aminoacids of *pcs*A gene. Aminoacids proposed to be involved in substrate binding are marked with a & symbol. Identity between *E. coli* citrate synthase and the deduced *pcs*A gene product is indicated by dots. Restriction sites: P, Pst1; B, BamH1; H, HindIII; and X, Xba1.

Fig. 2 A. a) Plasmid profile of strain CFN299. b) Autorradiogram of the same plasmid profile after hybridization with the *pcs*A BamH1-HindIII internal fragment. B. Autorradiogram of Southern blot EcoR1-digested genomic DNAs hybridized with the same BamH1-HindIII *pcs*A internal fragment. The 6 Kb hybridized band corresponds to the plasmid citrate synthase and the 9 Kb corresponds to the chromosomal citrate synthase gene. a) Wild type strain CFN299; b) CFNE299-10, a pSym spontaneous deletion mutant and c) CFNE130, a *pcs*A-Tn5 insertion mutant.

Fig. 3 Relationship between the complete *pcs*A gene and other citrate synthase genes. CisySPcsa, *pcs*A gene; CisySPseae, *P. aeruginosa*; CisySEcoli, *E. coli*; CisySAceac, *A. aceti*; CisySRicpr, *R. prowazekii*; CisySBacsp, *Bacillus subtilis*.

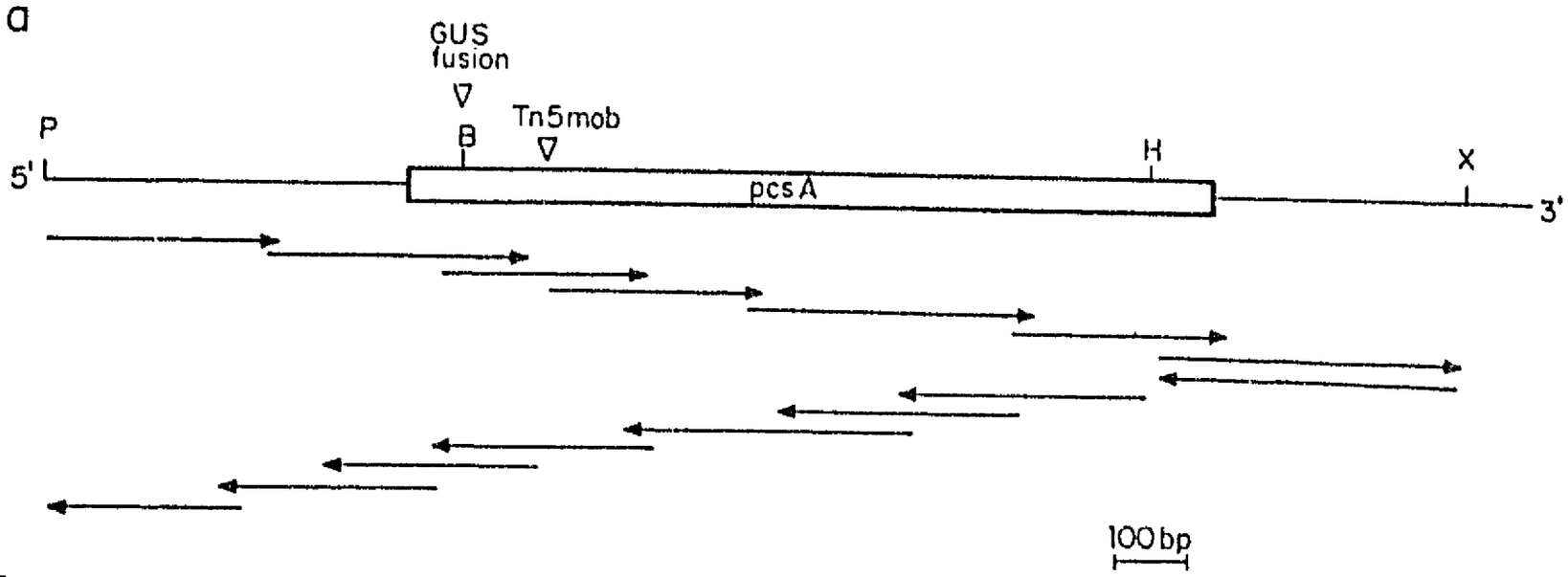
Fig. 4 A) Growth rate and B) Citrate synthase activity. Bacteria were grown in MM supplemented with 0.2% sucrose as carbon source. CFN299, *R. tropici* wild type strain; CFNE130, *pcs*A-mutant; CFNE137, a CFNE130 transconjugant that harbors a cloned *pcs*A gene; CFN42, a *R. leguminosarum* bv. *phaseoli* wild type strain; CFNE138, a CFN42 transconjugant harboring a cloned *pcs*A gene.

Fig. 5 Nodulation ability of CFN299, *R. tropici* wild type strain; CFNE130, *pcs*A-mutant; CFNE131, a CFNE130 rifampicin resistant; CFNE132, a CFNE131 with a wild type pSym; CFNE137, a CFNE130 that contains a cloned wild type *pcs*A gene; CFN42, *R. leguminosarum* bv. *phaseoli* wild type strain; CFNE138, a CFN42 that harbors the cloned *pcs*A gene.

Fig. 6. *pcs*A-gene expression determined as ß-glucuronidase activity from CFN299 grown in decreasing iron concentration. pMP6 plasmid was transferred to the wild type strain CFN299. The transconjugant was grown in MM with increasing amounts of the iron chelator 2-2'.. dipyridyl. The dotted line and (H) represent the condition where both 300 nM of dipyridyl and iron (1 µM) were added to the medium.

Fig. 7. Citrate synthase activity of (A) wild type strain CFN299, and (B) *pcs*A- mutant CFNE130, with increasing amounts of dipyridyl in the medium. As in fig. 6, the dotted line and (H) represent a medium condition where both 300 nM of chelator and 1μM of iron were added.

a



b

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CTGCAGATCGGCTCGACCCCGAACTTTGAGCGGTGTTCGTCGATGAAGGAAATCATCGCTTCAGTGGGCGGTCGAGCTCCGCTGGGCAAAATAAGCCGACGCCTTACGCA   110
AAATCTCATTGGCCTGTCGAAGCTCGCGGTTCTCCCGCTCAAGGGCCTTCATCTTCTCGGCGACATCGCTTGGAAGGCCTGCTCGTTTGCCGCTGTCAACATCGGCTTTC   220
TTCACCCACTCATGCAGCGTATGTGCCGAGCAGCCAATCTTGGCGCTATGGATGAAACTGCCGCCCACCGGATGGATGCTCGGCCTCATGGTCCAAAACCATGCGGATGG   330
CTCGGTCACGAACTTCAGGTGAAATTTGTTGGTCGTCTTGCTCATATCGGCTCCACTTTCTCACGAGTTGGAGCCTCCGCGAAACCCGGCGCGGTTCACCCCCTCTGCGG   440
GAAGACAATTAGAACACAGATCAGTTCATTCGGCAGCTGTTAGTTCCCAGCTAGCGAGCCCTGAGGAAAAATATCATGGATAACAACAATGCTTGTGTCTTAGTCGACGG   550
                                                                                  MetAspAsnAsnAsnAlaCysValLeuValAspGl
CCATAGTGCCGAATTGAAACTTCGATCAAGCACGATCGGTCCGAACGTCCTCGGCATTGGATCCCTCTACGAGCAGACGAAGATGTTCACCTATGATCCTGGCTTCACTT   660
12  yHisSerAlaGluLeuLysLeuArgSerSerThrIleGlyProAsnValLeuGlyIleGlySerLeuTyrGluGlnThrLysMetPheThrTyrAspProGlyPheThrS
CGACTGCGTCGTGCGAGTCCAGCATCACCTTTATTGATGGCGACGAAGGCGTTCTGCTGCATCGCGGTTATCCGATCGAACAGCTTGCCGAGCACGGCGACTTCCTCGAA   770
49  erThrAlaSerCysGluSerSerIleThrPheIleAspGlyAspGluGlyValLeuLeuHisArgGlyTyrProIleGluGlnLeuAlaGluHisGlyAspPheLeuGlu
GTCTGTTACCTGCTGCTCTACGGCGAATTGCCGACCGCAGCCCAGAAGAAGGACTTCGACTATCGGGTCGTGCACCACACCATGGTGCATGAACAAATGTCCCGCTTCTT   880
86  ValCysTyrLeuLeuLeuTyrGlyGluLeuProThrAlaAlaGlnLysLysAspPheAspTyrArgValValHisHisThrMetValHisGluGlnMetSerArgPhePh
CACCGGTTTCCGCCGCGATGCGCATCCGATGGCCGTCATGTGCGGCTGCGTCGGCGCTCTGTCGGCCTTCTATCACGACTCCACCGACATCACCGATCCGCACCAGCGCA   990
122 eThrGlyPheArgArgAspAlaHisProMetAlaValMetCysGlyCysValGlyAlaLeuSerAlaPheTyrHisAspSerThrAspIleThrAspProHisGlnArgM
TGGTCGCAAGCCTTCGTATGATCGCCAAGATGCCGACGCTTGCCGCCATGGCCTACAAGTACCATATCGGCAGCCCTTCGTTTACCCGAAGAACGATCTCGACTATGCG   1100
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GAACGCATCGACCTCGACGGTTCGCCTCGCCGGCTCTTCCGGCGCCAATCCCTTTGCTTGCATCGCCGCCGCATCGCATGCCTCTGGGGCCCGGCCCATGGCGGCGCCA   1320
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ACGAACGTGCGCTCAACATGCTGACGGAAATCGGCACGGTCGACCGCATTCCGGAATATATCGCCCGCGCAAGGACAAGAACGATCCGTTCCGATTGATGGGCTTCGGT   1430
269 snGluArgAlaLeuAsnMetLeuThrGluIleGlyThrValAspArgIleProGluTyrIleAlaArgAlaLysAspLysAsnAspProPheArgLeuMetGlyPheGly
CATCGCGTTTACAAGAACTACGATCCGCGCGCCAAGATCATGCAGAAGACGGCGCACGAGGTCCTCGGCGACTCGGCATCAAGGACGATCCGCTGCTCGACATCGCGAT   1540
306 HisArgValTyrLysAsnTyrAspProArgAlaLysIleMetGlnLysThrAlaHisGluValLeuGlyGluLeuGlyIleLysAspAspProLeuLeuAspIleAlaIl
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416 ThrGlyAlaProLeuArgGluTyrValProLeuSerLysArgEnd
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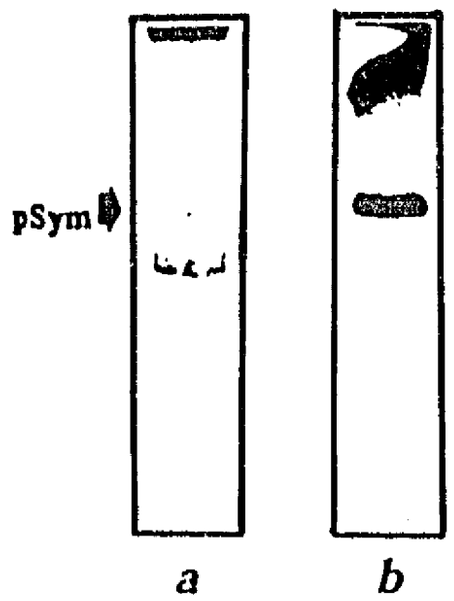
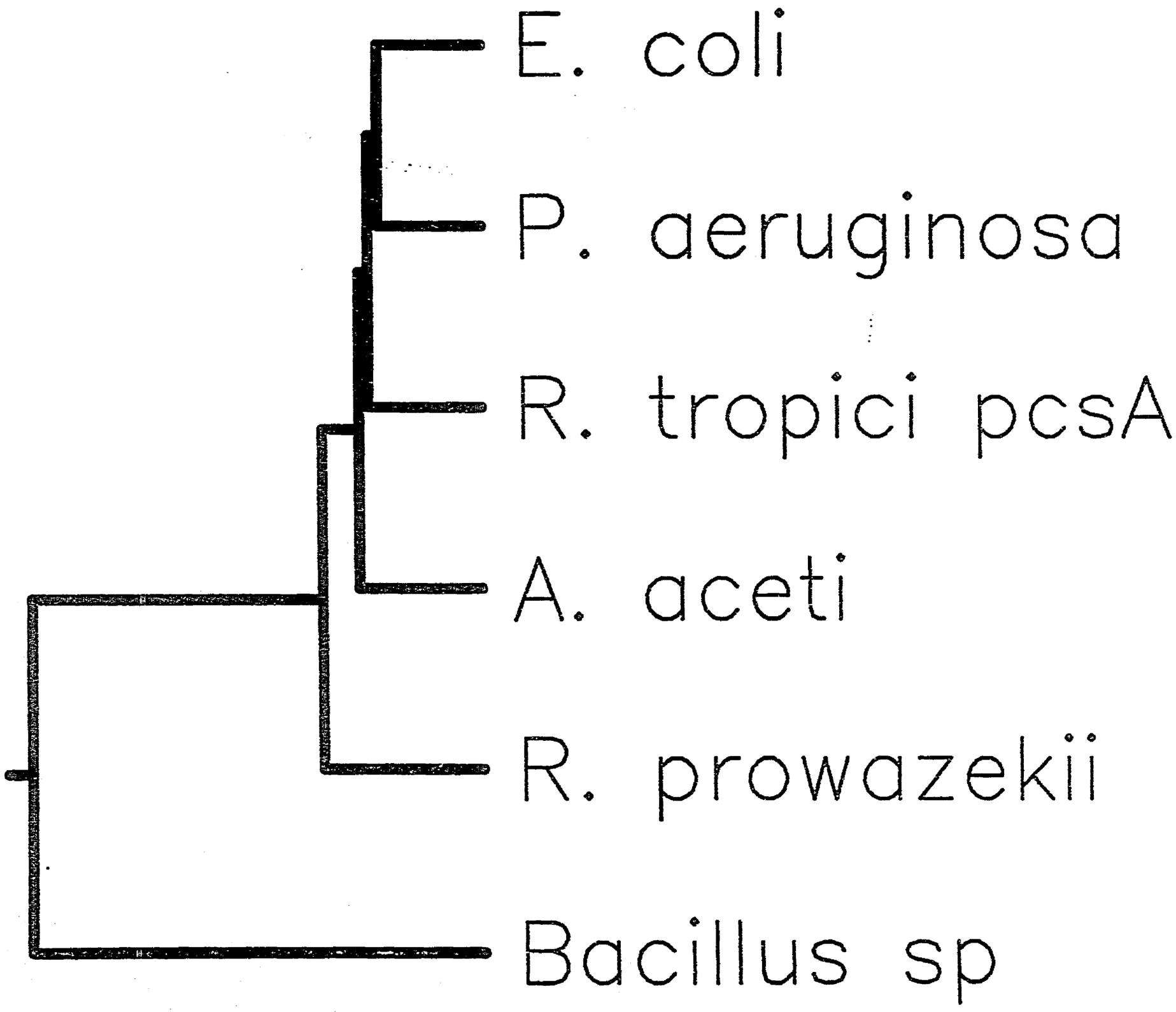
A



a b

B

a b c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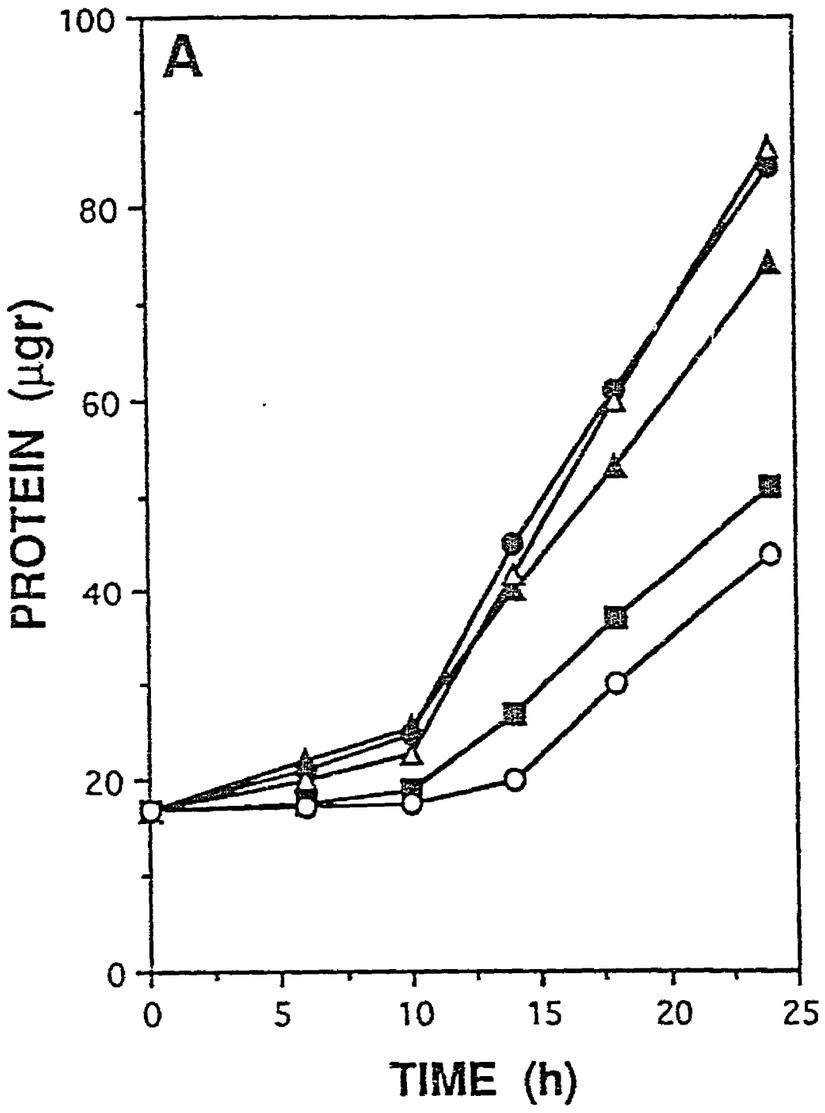
Bacillus sp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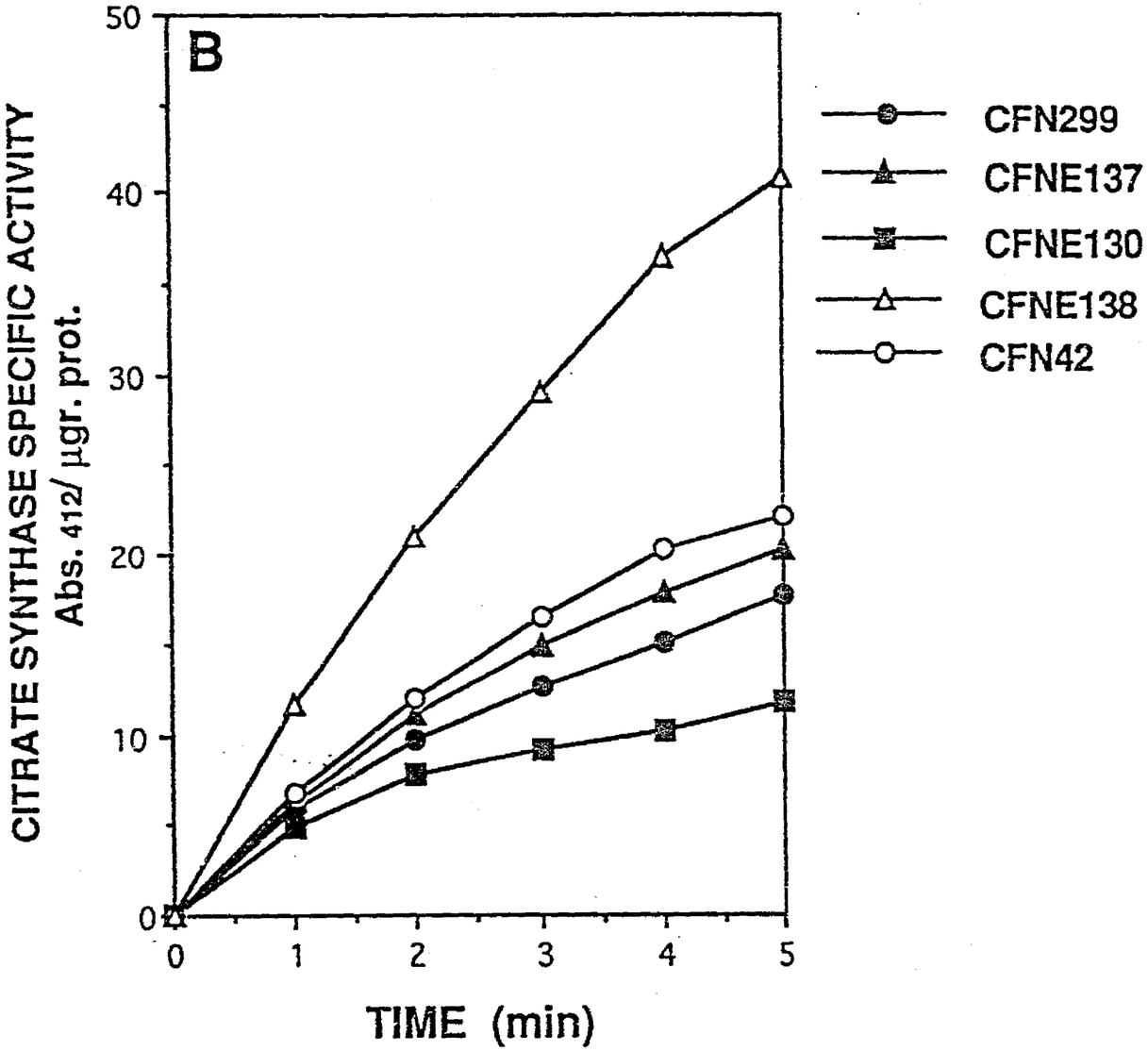
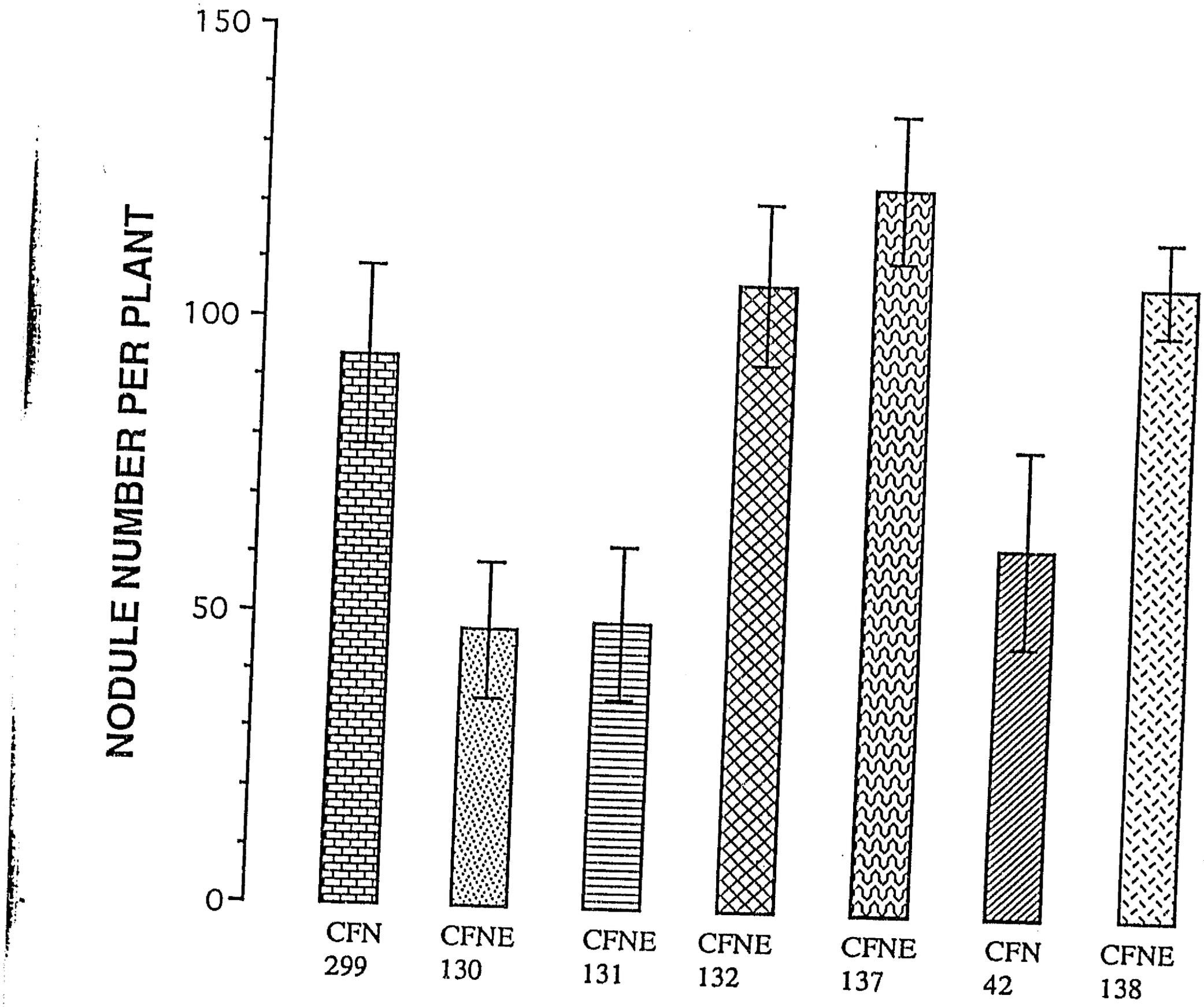
80
60
40
20
0
PROTEIN (µgr)
0
5
10
15
20
25
TIME (h)


B
50
40
30
20
10
0
CITRATE SYNTHASE SPECIFIC ACTIVITY
Abs. 412/ µgr. prot.
0
1
2
3
4
5
TIME (min)
CFN299
CFNE137
CFNE130
CFNE138
CFN42


NODULE NUMBER PER PLANT
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50
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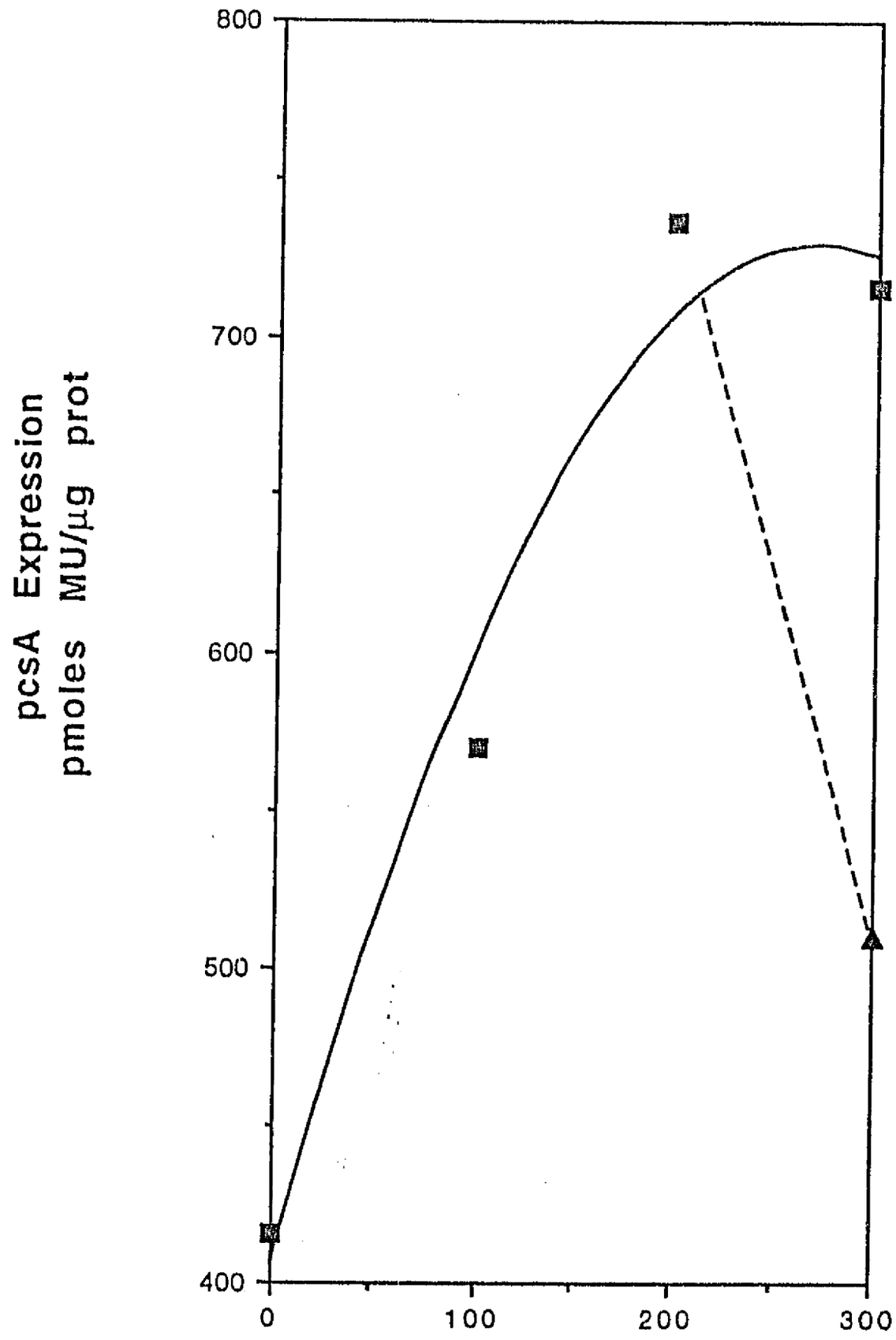
CFN 299
CFNE 130
CFNE 131
CFNE 132
CFNE 137
CFN 42
CFNE 138


800
700
600
500
400
0
100
200
300
pcsA Expression
pmoles MU/µg prot
2-2'-Dipyridyl (nmolar)

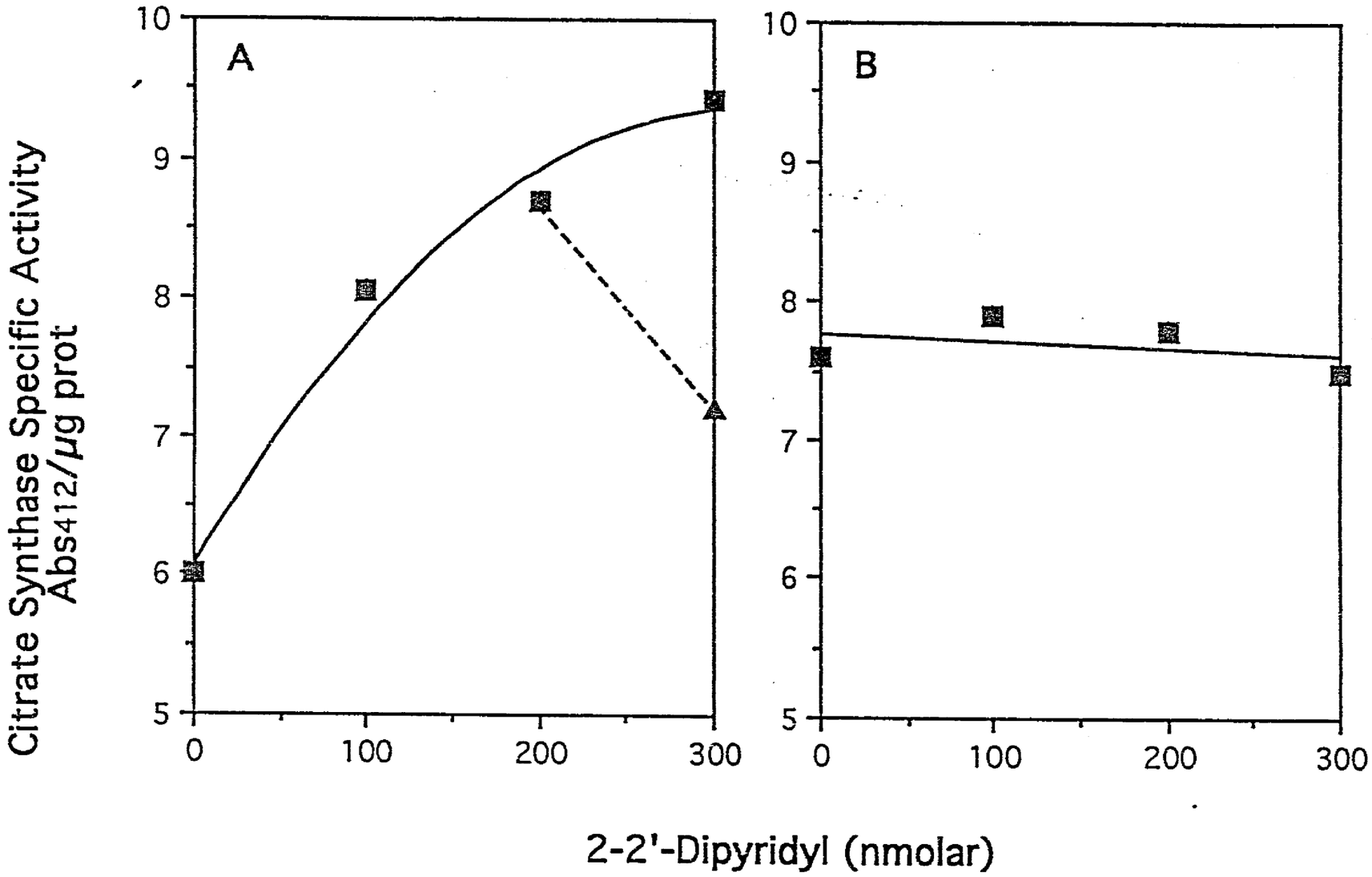
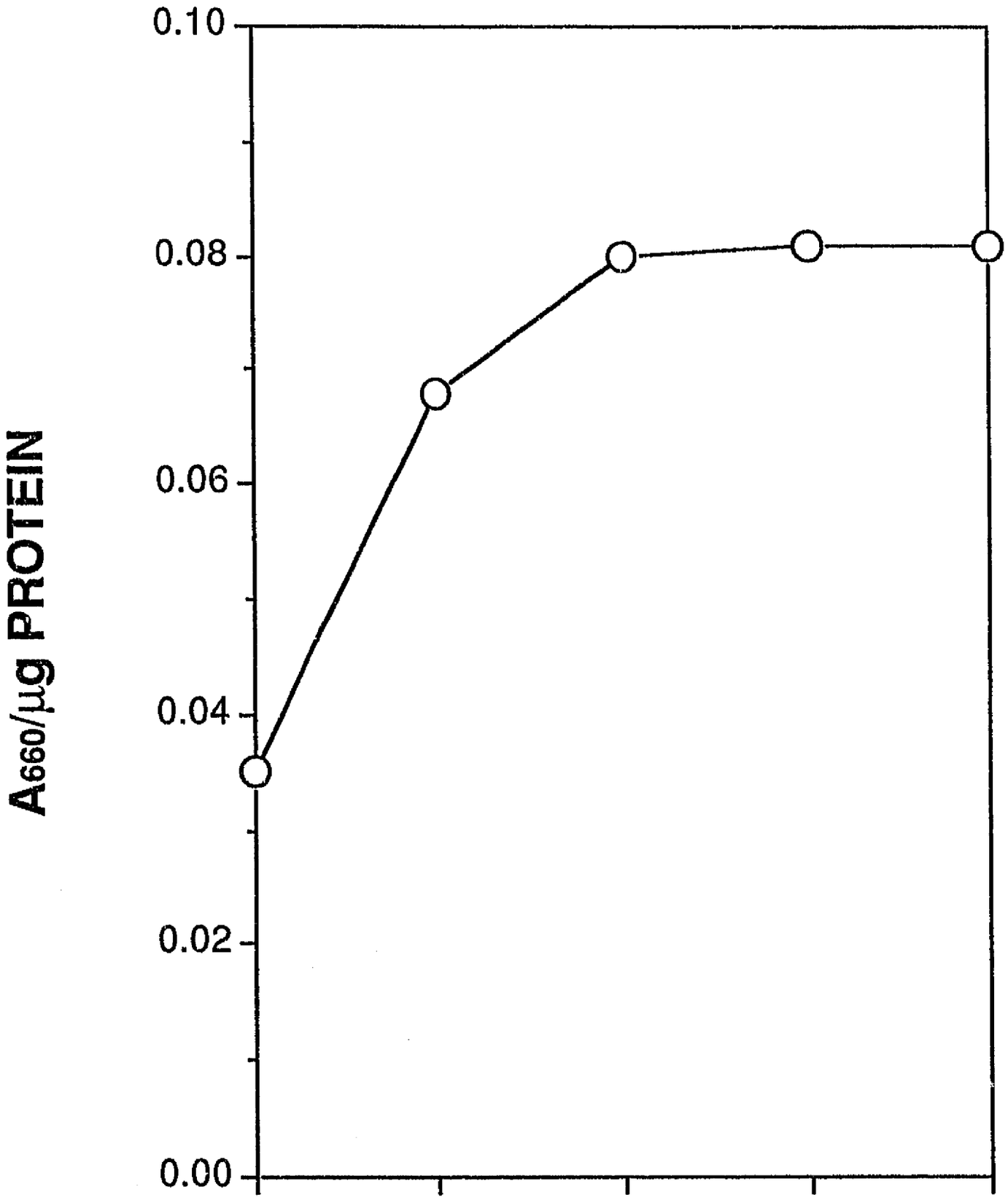

Citrate Synthase Specific Activity
Abs412/µg prot
A
B
10
9
8
7
6
5
0
100
200
300
2-2'-Dipyridyl (nmolar)

FIG. 8



CALCIUM DEPENDENCE OF *pcs* A GENE.

Discusión al primer trabajo

En este trabajo demostramos la presencia de un gene de citrato sintasa localizado en el plásmido simbiótico de *R. tropici*. Presentamos la secuencia nucleotídica del gene y su comparación con genes de citrato sintasa previamente reportados. Con base al fenotipo presentado por la mutante *pcs*A- y por experimentos de regulación génica, proponemos que la citrato sintasa plasmídica juega un papel en los procesos celulares de 1) capacidad de crecimiento en sacarosa, 2) captación de los iones hierro y calcio y 3) como consecuencia de los dos anteriores, en su capacidad simbiótica (eficiencia de nodulación).

Cuando un nuevo caracter se fija en la población de una determinada especie, es porque le confiere alguna ventaja adaptativa sobre su medio ambiente. ¿Qué ventajas le confiere entonces la citrato sintasa plasmídica a *R. tropici*? Creemos que por lo menos dos: 1) Le permite adquirir elementos químicos poco accesibles en el suelo como son el hierro y el calcio, importantes para el *Rhizobium* tanto en vida libre como en simbiosis y 2) le confiere una mayor capacidad de crecimiento a la bacteria en presencia de sacarosa.

Con respecto a la primera, la citrato sintasa produciría cítrico que se excretaría al medio para atrapar (quelar) hierro y calcio. Esto es factible si consideramos que *R. tropici* proviene de suelos ácidos, en donde el hierro se encuentra en forma soluble. El ácido cítrico es un quelante de baja afinidad comparado a otros sideróforos (14,50). En condiciones ácidas del suelo no se requieren sideróforos de alta afinidad y además, para la bacteria es muy barato sintetizar ácido cítrico comparado a lo requerido para sintetizar sideróforos de alta afinidad, necesarios probablemente para cuando el hierro sea químicamente poco accesible o bien sea muy escaso.

No sólo se induce la expresión del *pcs*A ante una limitación de hierro, sino que también responde a la limitación de calcio (fig. 8, en el anexo). El calcio es un elemento esencial para todo organismo. En suelos semisecos o secos el calcio es abundante, no así en suelos húmedos. Los suelos húmedos lixivian al calcio y el disminuir su concentración contribuye a la acidificación del suelo. Recordemos que *R. tropici* proviene de suelos ácidos, esto es, de suelos donde el calcio es escaso. Es probable entonces que la bacteria excrete cítrico con el fin de atrapar el poco calcio disponible en estas circunstancias. Existe la posibilidad de que la

contribución de la citrato sintasa plasmídica en el atrapamiento de hierro y calcio se refleje durante la vida de la bacteria fuera de la planta y no en su relación simbiótica. Apoya esta posibilidad el hecho de que la eficiencia de nodulación en la CFNE130 no se restablece a los niveles de la silvestre agregando hierro y calcio al medio nutritivo de la planta (datos no presentados).

La segunda ventaja evidente es la capacidad de crecer más rápido en sacarosa. En la rhizósfera, las raíces excretan varios azúcares como son glucosa, fructosa, maltosa, ribosa,arabinosa, algunas sacarosa y ácidos orgánicos como glutamina, aspártico, triptofano y otros (91). Podría pensarse que *R. tropici* , al poseer la citrato sintasa plasmídica, colonizaría más rápidamente la superficie radicular y por ende tendría mayores posibilidades de infectar la planta. Sin embargo, la cantidad de nódulos que son capaces de formar tanto la cepa silvestre como la CFNE130 se mantiene constante si se inoculan plantas a baja concentración bacteriana (1X10*3/ml) o a alta (1X10*6/ml), lo que sugiere que el mejoramiento de la eficiencia de nodulación por la presencia del *pcs*A no depende de una alta población en la rhizosfera, sino de un eficiente crecimiento en el hilo de infección. Por otro lado sabemos que el número de nódulos se determina en las primeras horas del proceso simbiótico y es la planta la principal responsable a través del proceso de *autorregulación* (66). Durante este proceso la planta inhibe el desarrollo de la gran mayoría de los focos infectivos. La bacteria por su parte debe desplazarse rápidamente a través del hilo de infección (o por los espacios intercelulares) e invadir células meristemáticas del foco antes de que el desarrollo de éste sea inhibido por la planta. Ahora bien, si consideramos que la bacteria recibe durante su recorrido compuestos carbonados del floema y, en el caso del frijol y en la mayoría de las plantas, éste consiste principalmente de sacarosa (28); y si tomamos en cuenta que el *pcs*A le confiere a la bacteria la posibilidad de crecer más rápido en sacarosa, entonces es probable que el *Rhizobium* se desplaze por el hilo de infección más rápido, infecte más focos, dándole así posibilidades de lograr un mayor número de nódulos. Esto se puede comprobar si comparamos el número de focos invadidos por la silvestre en relación con los de la mutante CFNE130 en una unidad de tiempo, donde esperamos sean menos en la mutante. Un dato fuerte que favorece esta hipótesis es el obtenido con la cepa CFNE138, la cual es un *R. etli* al que se le ha transferido el *pcs*A. Esta tranconjugante crece más rápido en sacarosa, y su eficiencia de nodulación mejora notablemente (ver fig. 4 y 5).

Es común la presencia de secuencias génicas reiteradas en *Rhizobium* (30). Una de las interrogantes que ha generado este trabajo es aquélla que concierne al cómo se generó el *pcs*A, tal que se mantiene casi idéntica la secuencia codificadora de la citrato sintasa plasmídica (gene estructural) con respecto a la cromosomal (I. Hernández,tesis de Lic.), pero contiene una región regulatoria diferente (decimos región regulatoria diferente porque, a diferencia de la plasmídica, la actividad de la citrato sintasa cromosomal no se modula en relación a la asequibilidad de hierro y calcio. Actualmente esta región es analizada a nivel de su secuencia de nucleótidos por I. Hernández). Una posible explicación sería considerar la existencia de transcriptasa reversa en *Rhizobium* (40). La transcriptasa reversa sintetiza DNA a partir de un templado de RNA. Esto significa que si el templado es un RNA codificador de una proteína, en principio la enzima es capaz de sintetizar al gene correspondiente. Después, a través de algún proceso recombinatorio, este gene podría integrarse en algún sitio del genoma próximo a una región regulatoria y que, por las características de regulación de esta región, esta quimera se haya seleccionado favorablemente.

No es improbable que el origen del *pcs*A haya sido externo, posiblemente a través de un evento de transposición. Arriba del *pcs*A (upstream) se localiza un secuencia en fase (ORF) semejante a un gene contenido en la secuencia de inserción IS1 de *E. coli*, específicamente de la transposasa. La presencia de esta secuencia sugiere la posibilidad de que el *pcs*A (y sus regiones adyacentes) se hayan insertado en el plásmido simbiótico por transposición. Sería recomendable buscar secuencias hacia arriba y hacia abajo del *pcs*A que pudieran formar parte de un transposón (como secuencias invertidas repetidas).

Antecedentes particulares al segundo trabajo

La relación simbiótica del *Rhizobium* con la planta conlleva ajustes metabólicos y genéticos en ambos simbiontes. Estos ajustes deben coordinarse en el tiempo y el espacio, y para ello tanto la bacteria como la planta utilizan un sistema de comunicación. Es importante resaltar que los participantes de este proceso simbiótico pertenecen a organismos muy distintos: el *Rhizobium* es un procariote y la planta un eucariote. Esto significa que sus mecanismos de regulación genética y/o metabólica son de inicio diferentes. A pesar de ello, se ha generado un lenguaje a través de señales químicas en ambos sentidos que permite una comunicación exitosa y que consecuentemente culmina en un beneficio mutuo.

Uno de los intereses en nuestro grupo es el de conocer y estudiar la naturaleza de estas señales en *Rhizobium*, así como el cómo, cuándo, dónde y para qué se expresan.

Ya se mencionó que la planta excreta ciertos compuestos flavonoides que la bacteria capta y que estimulan la expresión de genes bacterianos necesarios para la nodulación (genes nod). Esta primera señal es de naturaleza selectiva, esto es, sólo un *Rhizobium* o un pequeño grupo de rhizobia responderá a ella. Los genes nod, a su vez, codifican a enzimas involucradas en la síntesis de los factores de nodulación (factores Nod), que son lipo-oligosacáridos que inducen cambios morfológicos en la raíz de la planta. Estos lipo-oligosacáridos son la primera señal que envía el *Rhizobium* a la planta. Este morfógeno induce la deformación del pelo radicular y la formación del foco infectivo o primordio, constituído por células meristemáticas de la corteza y que darán lugar a la formación del nódulo. Los factores Nod *per se* son capaces de inducir estructuras tipo nódulo en la raíz (94).

La organogénesis en plantas se dá controlando los niveles relativos de distintas fitohormonas (84). Mucho se ha especulado acerca del papel de las fitohormonas en la organogénesis del nódulo (6,99), y específicamente del papel de las fitohormonas producidas por la bacteria. Esta inquietud no es gratuita debido a que otros sistemas bacterianos que interactúan con plantas, como *Agrobacterium tumefaciens* y *Pseudomonas savastanoi*, alteran los niveles relativos de hormonas en la planta como parte del proceso infectivo (2,12). Varios investigadores han tratado infructuosamente de descubrir una correlación entre las fitohormonas

producidas por *Rhizobium* y la simbiosis (5,80). Hirsch y colaboradores (ver ref.6 del segundo trabajo) mostraron que inhibidores del transporte de auxinas inducen la formación de estructuras tipo nódulo en las raíces de alfalfa. Esto nos sugiere que el ácido indolacético juega un papel, o varios, en la génesis del nódulo.

Sabemos que *Rhizobium*, como otros microorganismos del suelo, en cultivo producen bajas cantidades de ácido indolacético. Esta producción basal parece no tener un papel en la relación planta-*Rhizobium* por lo que, para el inicio de este trabajo, propusimos que en *Rhizobium* se induce la producción de indolacético en presencia de exudados de la planta. La propuesta se establece considerando que la producción del factor de nodulación, morfógeno indispensable en la génesis del nódulo, se induce en presencia de estos exudados.

En este trabajo se presenta la inducción de la producción de ácido indolacético (IAA) en *Rhizobium tropici* por flavonoides de la planta y una correlación entre esta producción de IAA y la nodulación.

INDOLEACETIC ACID PRODUCTION IS INDUCIBLE BY FLAVONOIDS IN *Rhizobium tropici*.

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RUNNING TITLE:

Induced Indoleacetic Acid Production in *Rhizobium*

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ABSTRACT

Indoleacetic acid (IAA) production is enhanced several fold in *Rhizobium tropici* when specific flavonoid compounds are present in the medium. This inducible IAA production system is pSym-encoded and it is regulated by flavonoids at the transcriptional level. A pSym mutant unresponsive to flavonoid induction has a diminished nodulation efficiency.

The successful infection of leguminous plants by *Rhizobium* and *Bradyrhyzobium* results in a symbiotic association that enables the reduction of atmospheric dinitrogen to ammonia. *Rhizobium* infects root cortical cells and causes the formation of a tumor-like structure, the nodule, that provides the micro-environment necessary for the transformation of *Rhizobium* into a nitrogen-fixing bacteroid.

Previous studies suggest that plant hormones participate on the induction of nodule primordium. Libbenga et al. (11) have shown that auxins and cytokinins induce cell divisions in pea root explants at sites similar to those of nodule primordium formation. Moreover, auxin-transport inhibitors induce nodule-like outgrowths on the roots of alfalfa (6).

. Auxins, mainly IAA, have an important role in the organogenesis of plants. Thus, IAA has been shown to induce or stimulate stem and root cell elongation, cell division, xylem and phloem differentiation, adventitious root formation, and many other processes (21).

Not only plants synthesize IAA, but also many microorganisms. Some bacteria that interact with plants, such as *Agrobacterium* and *Pseudomonas*, produce IAA as part of their infection strategy (1,4).

Because phytohormones are viewed as major elements in the regulation of plant growth and differentiation, a substantial research effort has been expended to explore the possibility that phytohormones produced by *Rhizobium* have a role on nodule development. Supportive evidence is largely circumstantial, based on demonstrations of the production of IAA, cytokinin and giberrellin-like substances by *Rhizobium* in culture. Previous research has failed to demonstrate a correlation between the ability of *Rhizobium* to produce IAA and to nodulate peas and clover (3). Other workers could not demonstrate a correlation between IAA production and certain mutants defective in nodulation and nitrogen fixation (2).

In *Rhizobium* species, the majority of the nodulation (*nod*) genes lie in a large symbiotic plasmid (pSym). The common *nod* genes, *nod*ABC, are found in all rhizobia and they are required for the biosynthesis of the core molecule of lipo-oligosaccharide Nod factors that deform root hairs and initiate host cell proliferation (10,20). Other loci, the host-specific (hsn) *nod* genes, encode enzymes that modify the basic Nod factors so that they deform root hairs and elicit cortical cell divisions in a host-specific manner (17). It has been shown that specific flavonoid compounds present in plant root exudates are required for the coordinated expression of early *nod* genes (12,14,15).

Nodulation genes are under the positive control of the regulatory gene *nod*D, which product is believed to be activated by flavonoid signals from the host and then to complex with *cis*-regulatory elements termed *nod* boxes located upstream of the *nod* genes (7,8,16).

We hypothesized that if there were an IAA production system in *R. tropici* involved in the symbiotic process, it might be regulated by flavonoids as other nodulation genes. In this paper we report a flavonoid-inducible IAA production (FIIP) system in *R. tropici*, Sym plasmid borne, that may have a role in nodulation efficiency.

IAA production by *R. tropici*. *R. tropici* strain CFN299 (wild type) was grown in MM supplied with tryptophan in the presence or absence of different flavonoid compounds. After 15 hr of growth, cells were harvested and the supernatant treated for IAA extraction and quantification. The acidic indole fraction was extracted with ethyl acetate, dried under nitrogen flux and resuspended in methanol. A thin-layer chromatography was carried out and the IAA migration region was taken. IAA was extracted with methanol and runed in HPLC for quantification. Results are shown in Fig. 1. IAA production is enhanced several fold when different flavonoid compounds are present in the medium. It is noteworthy that the same pattern of induction by flavonoids is obtained for the production of nodulation factors in *R. tropici* (14).

Since flavonoid-dependant *nod* genes are mainly found in the symbiotic plasmid of *Rhizobium* species, it was interesting to find out if the FIIP system was also Sym plasmid borne. A Nod— 200-Kb spontaneous pSym deletion mutant (CFNE299-10) was tested for induced IAA production. IAA production in this mutant is not enhanced by flavonoid compounds (Fig.1), which clearly indicates that at least part of the FIIP system is pSym encoded.

Regulation of the FIIP system. *R. tropici* CFN299 was mutagenized with Tn5*gus*A (19) to obtain transcriptional fusions which allow gene-transcription analysis by measuring ß-glucuronidase activity (9). Tn5*gus*A insertions located on the symbiotic plasmid were selected by individually mating all the CFN299 mutants with a plasmid-free *Agrobacterium tumefaciens* strain GMI9023 (18). *Agrobacterium* transconjugants bearing the symbiotic plasmid were selected by plasmid visualization using the Eckhardt procedure (5). 400 parental pSym insertion mutants were tested for IAA production in response to flavonoid compounds. One of these mutants, CFNE134, was found to be no longer responsive (Fig.1). Interestingly, apigenin-stimulated IAA production was

unaffected in this mutant, suggesting an alternative regulatory pathway for induced IAA production or an alternative inducible IAA biosynthetic pathway.

Growth conditions and inducers were the same as those used for IAA production experiments. Gene expression results are shown in Table 1. The sequence fused with the transposon in mutant CFNE134 is transcriptionally regulated by flavonoid compounds, and the inducers are the same as those for IAA stimulated production. It is possible then that the Tn5*gus*A interrupted sequence of mutant CFNE134 could be regulated through the *nod*D product as other *nod* genes. Additional studies are necessary to confirm this possibility.

The FIIP system and the symbiotic process. Mutant CFNE134 was tested for its nodulation phenotype in *Phaseolus vulgaris*. Nodulation assays were performed in agar flasks and in vermiculite jars using *P. vulgaris* Negro Jamapa as described in Martínez et al., 1985. (13). After 15 days, nodule number per plant was determined. Nitrogenase activity was measured by acetylene reduction.

Nodules formed by this mutant were undistinguishable from those of the wild type, its specific nitrogenase activity was similar, there was no delay in nodulation and no change in host specificity (data not shown). However, nodulation efficiency was clearly affected in the mutant strain, showing a reduced nodule number capacity of around 50-60% of that of the wild type (Fig.2).

The Tn5-*gus* mutated region of CFNE134 was cloned and its partial nucleotide sequence has been obtained. It reveals neither homology to *nod*D nor to other reported *nod* genes (not shown).

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FIG. 1

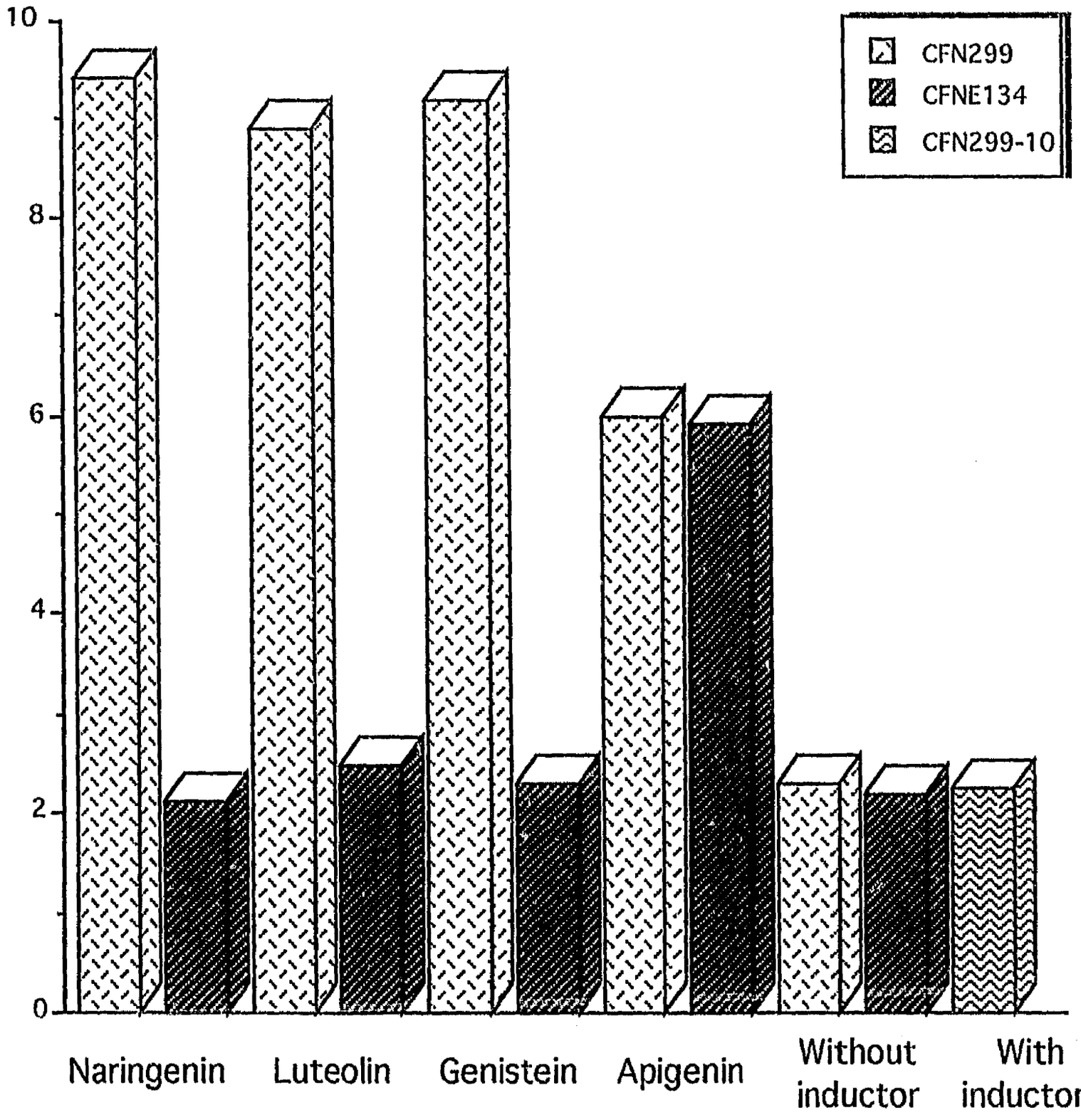
10
8
6
4
2
0
ng IAA/µg PROTEIN
CFN299
CFNE134
CFN299-10
Naringenin
Luteolin
Genistein
Apigenin
Without inductor
With inductors

FIG. 2

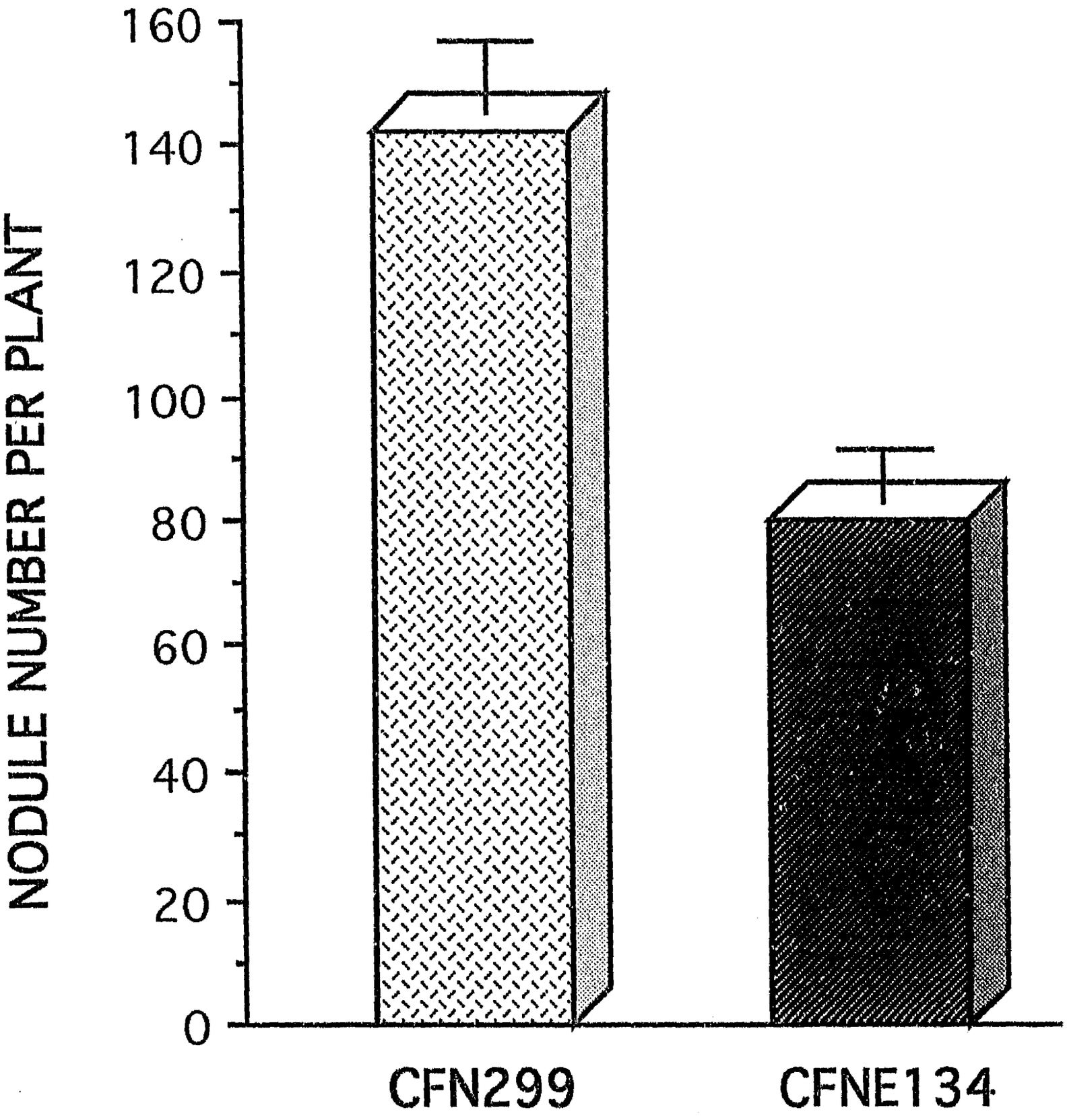
NODULE NUMBER PER PLANT
160
140
120
100
80
60
40
20
0
CFN299
CFNE134

TABLE 1. Relationship of induced IAA production and *gus*A gene expression in CFNE134

INDUCER	INDUCTION OF IAA PRODUCTION		ß-glucuronidase activity of CFNE134
	CFN299	CFNE134	
Naringenin	+	-	+
Genistein	+	-	+
Luteolin	+	-	+
Chrysin	+	-	+
Apigenin	+	+	+
Quercitin	-	-	-
Exudate	n.t.	n.t.	+

n.t. = not tested

Fig.1.- Indoleacetic acid production of *R. tropici* CFN299, CFNE134, and CFNE299-10. In the corresponding condition, inducer concentrations were as follows: 10 µM luteolin, 0.8 µM naringenin, 0.8 µM genistein and 0.8 µM apigenin. CFNE299-10 was tested with all four inducers. Tryptophan was added to the growth medium at a final concentration of 0.4 mg/ml.

Fig.2.- Nodulation efficiency of CFN299 (wild type strain) and CFNE134 mutant in *Phaseolus vulgaris*. Nodule number was determined 15 days after inoculation.

Discusión al segundo trabajo

En este trabajo hemos demostrado una correlación entre la producción de ácido indolacético por parte del *Rhizobium* y el proceso de nodulación. Hemos descrito un sistema de producción de IAA inducible por compuestos flavonoides (FIIP), que reside en el plásmido simbiótico y que afecta la eficiencia de nodulación. El sistema FIIP no es esencial para la formación del nódulo *per se*, sino que incide sobre la capacidad de la bacteria en lograr un mayor número de simbiosis.

En *Rhizobium*, la expresión de los genes nod es dependiente de compuestos flavonoides específicos exudados por la planta. En este trabajo hemos demostrado que el sistema FIIP presenta el mismo patrón de inducción por flavonoides que el descrito para la producción del factor Nod en *R. tropici*, lo cual sugiere que el sistema FIIP está sujeto a un control genético común con los genes nod. Probablemente se encuentre bajo un control transcripcional vía nodD. Demostrar esta posibilidad no es fácil dado que se ha visto que *R. tropici* posee cinco genes nodD (98). Sin embargo, se podrían realizar experimentos analizando uno por uno en un sistema FIIP aislado (por ejemplo en un *Rhizobium* desprovisto de plásmidos) y de esta manera observar la contribución de cada *nod*D a la regulación del sistema FIIP.

Existe, sin embargo, la posibilidad de que el sistema FIIP sea regulado además por otros factores diferentes a los flavonoides. Los genes *nod* sujetos a la regulación por nodD se expresan sólo durante las etapas tempranas de la nodulación, no así el sistema FIIP el cual hemos observado, al menos en la secuencia interrumpida de la mutante CFNE134, que se expresa en el nódulo maduro (Fig. A1).

¿Sobre qué proceso o procesos de la simbiosis incide el sistema FIIP? ¿Será su función alterar los niveles hormonales de la planta en el sitio de infección? ¿Tendrá una función con respecto al proceso de autorregulación de la planta? Estas y otras interrogantes tendrán que ser despejadas una vez se tenga más información acerca de los genes involucrados en el sistema FIIP. Su secuencia nucleotídica nos dará información acerca de la posible actividad de sus productos y de regiones involucradas en la regulación de su expresión génica; un análisis de su regulación nos hablará acerca de su papel simbiótico, como lo harán también las alteraciones genéticas (sistema FIIP multicopia, el sistema FIIP en un fondo genético distinto, etc.).

FIG. A1

a. Actividad de ß-glucuronidasa en nódulos de frijol formados por la cepa CFN299 y la mutante CFNE134.

b. Actividad de ß-glucuronidasa en corte de nódulo formado por la mutante CFNE134.

Será interesante colocar al sistema FIIP en un *Rhizobium* diferente y observar su comportamiento y repercusión en el proceso simbiótico, ya que no hemos encontrado que otros rhizobia, como *R. etli* o *R. meliloti*, aumenten su producción de IAA en presencia de flavonoides. Muy recientemente, un grupo del Japón, en comunicación personal, han encontrado que *Bradyrhizobium elkanii*, mas no *B. japonicum*, aumenta su producción de IAA en presencia de exudados. Estos resultados sugieren que el sistema FIIP es una adquisición reciente. Es interesante mencionar en este punto que el pariente más cercano de *R. tropici* es *Agrobacterium rhizógenes*, el cual produce IAA y enzimas que actúan en la desconjugación de auxinas como parte de su estrategia infectiva. Pudiera ser que tanto *A. rhizogenes* como *R. tropici* compartieran, dada su cercanía, algunos genes o estrategias de infección. Será interesante saber si *A. rhizógenes* comparte, al menos en alguno de sus componentes, al sistema FIIP de *R. tropici*.

Puesto que la simbiosis *Rhizobium*-planta ha resultado ser un proceso más exquisito y complejo que otros sistemas infectivos en los cuales la bacteria produce IAA (como *A. tumefaciens*), no esperamos que el *Rhizobium* necesariamente manipule hormonalmente a la planta de la misma manera ni con el mismo propósito que lo hacen los patógenos. Los factores Nod son un ejemplo y se propone que probablemente alteren, de manera directa o indirecta, los niveles relativos de hormonas en el sitio de infección (29). El sistema FIIP bien pudiera ser un control fino de estos niveles hormonales.

El anterior estudio está centrado en la producción de IAA. Sin embargo, *Rhizobium* también produce basalmente otras fitohormonas, como citocininas y giberelinas (5). Será interesante hacer un estudio similar con respecto a estos reguladores del crecimiento.

Discusión general

Las interacciones entre microbios del suelo son complejas. Es crucial poder predecir y/o mejorar la supervivencia y competitividad de microorganismos del suelo que sean benéficos para la economía y la ecología. Crucial para resolver problemas como el control biológico de enfermedades en plantas y el mejoramiento de la fijación biológica de nitrógeno en simbiosis.

En relación a la fijación biológica de nitrógeno en simbiosis, el problema de la competencia por nodular ha resultado ser la limitante para la introducción de cepas de *Rhizobium* al campo. Cepas de *Rhizobium* con claras ventajas de nodulación o fijación de nitrógeno en el laboratorio han tenido cierto éxito cuando son usadas como inoculantes en suelos donde existe una baja población de rhizobia autóctona. Sin embargo, cuando la población autóctona es alta, las cepas de laboratorio son incapaces de competir con ellas, aún en altas concentraciones de inoculante. De hecho, se ha demostrado que la respuesta al inoculante es inversamente proporcional al tamaño de la población de rhizobia indígena (92). Esto significa que los rhizobia autóctonos están bien adaptados a su nicho y que excluyen, por ventajas aún desconocidas, a las cepas introducidas. Aún no hemos comprendido cuáles son los factores importantes en la competencia por ocupar un nódulo, aunque se han propuesto ciertas características como antibiosis, motilidad y velocidad de nodulación (93,10,56). Lo cierto es que la competencia por nodulación pareciera ser un grupo de características que, en su conjunto, confieren a la bacteria un grado de competitividad por ocupar un nódulo.

Uno de los factores que definen al concepto de competencia es la eficiencia de nodulación, esto es, la capacidad individual de una cepa de lograr un mayor número de simbiosis. Se han descrito mutantes de *Rhizobium* afectadas en eficiencia de nodulación (vr. gr. exoG en *R. meliloti* y exoB en *B. japonicum* (63,71 ver ref.). Estas mutantes siempre van acompañadas de otra alteración, como son un retraso en la nodulación o un problema en la fijación de nitrógeno. En la presente tesis describo dos mutantes, CFNE130 y CFNE134, aparentemente no relacionadas, alteradas en su eficiencia de nodulación.y no en alguna otra característica simbiótica (faltaría llevar a cabo experimentos para determinar la cinética de nodulación). Estas mutantes ubican entonces a la eficiencia de nodulación como un caracter independiente y multifactorial. Será interesante buscar

otros genes que afecten la eficiencia de nodulación , incluso genes cromosomales. Por ejemplo, en *Rhizobium*, la fijación de nitrógeno está determinada por genes codificados en plásmido y por genes cromosomales (como serían los genes involucrados en el transporte y asimilación de ácidos dicarboxílicos). Esto con el fin de obtener un cuadro general de las funciones celulares importantes para la eficiencia de nodulación.

R. tropici posee características simbióticas comunes con otros rhizobia, pero también características particulares. La citrato sintasa plasmídica y el sistema FIIP son un ejemplo, ya que sólo han sido encontrados en *R. tropici* (con la posibilidad de que el sistema FIIP se encuentre en *B. elkanii*). Pareciera que para establecer una relación simbiótica con la planta, se ha seleccionado una estrategia básica común a los rhizobia (como sería la producción del factor Nod) y, para cada especie, una serie de modalidades particulares que le permiten adaptarse a su nicho y establecer con su macrosimbionte respectivo, una relación simbiótica más exitosa.

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ANEXO

INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, July 1991, p. 417–426 Vol. 41, No. 3
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Rhizobium tropici, a Novel Species Nodulating *Phaseolus vulgaris* L. Beans and *Leucaena* sp. Trees

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A new *Rhizobium* species that nodulates *Phaseolus vulgaris* L. and *Leucaena* spp. is proposed on the basis of the results of multilocus enzyme electrophoresis, DNA-DNA hybridization, an analysis of ribosomal DNA organization, a sequence analysis of 16S rDNA, and an analysis of phenotypic characteristics. This taxon, *Rhizobium tropici* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli (type II strains) and was recognized by its host range (which includes *Leucaena* spp.) and *nif* gene organization. In contrast to *R. leguminosarum* biovar phaseoli, *R. tropici* strains tolerate high temperatures and high levels of acidity in culture and are symbiotically more stable. We identified two subgroups within *R. tropici* and describe them in this paper.

Members of the genus *Rhizobium* nodulate the roots of leguminous plants. The rhizobia that infect peas, clovers, and beans (*Phaseolus vulgaris* L.) are clustered in a single species, *Rhizobium leguminosarum* (29), which has three biovars (*Rhizobium leguminosarum* biovar viciae, *Rhizobium leguminosarum* biovar trifolii, and *Rhizobium leguminosarum* biovar phaseoli); these biovars contain different symbiotic plasmids that encode distinct nodulation specificities. Nevertheless, heterogeneity in *Rhizobium leguminosarum* biovar phaseoli has been identified by using such different criteria as protein pattern (50), antibiotic resistance (2), serological type (49), multilocus enzyme electrophoresis behavior (45), DNA-DNA hybridization data (10, 26, 54), plasmid profile (37), and exopolysaccharide structure (70).

We previously distinguished two different types among isolates obtained from bean nodules and found differences in their symbiotic plasmids (36, 38, 39). Type I strains have multiple copies of nitrogenase *nifH* genes (39, 46), a narrow nodulation host range, and hybridize with the *psi* (polysaccharide inhibition) gene (3). Type II strains have single copies of *nif* genes, nodulate *Leucaena* spp., and do not hybridize with the *psi* gene (36, 39).

Type II strains have received attention because their symbiotic plasmids promote an effective and completely differentiated symbiotic process in *Agrobacterium tumefaciens* recipients (5, 38). They are genetically stable, retaining their symbiotic plasmid after prolonged incubation at 37°C. Some are heat tolerant (31) or acid and aluminum resistant (12, 25, 30, 62). The nodulation genes from one of these strains have been cloned (64). The chemical composition and structure of the extracellular polysaccharides from one type II strain differ from the chemical composition and structure of the extracellular polysaccharides from type I isolates (23).

Type II strains have been less successful in competition for bean nodule occupancy than the type I strains used (41). The former have been reported to occur less frequently in bean nodules (39). Nodule occupancy by type II strains can be enhanced under acid conditions (47, 63).

To define the taxonomic position and the genetic relatedness of type II strains, we analyzed 64 type II strains having different geographical origins and compared them with other species of rhizobia.

For a long time multilocus enzyme electrophoresis has been a standard method used in systematics (44), and this method is perhaps the best approach in large-scale studies to estimate the genetic diversity and structure of related populations (55, 67, 68). The results of multilocus enzyme electrophoresis studies provided the basis for the identification of two previously undescribed species among *Legionella pneumophila* strains (57) and identified two groups of bacteria within *Rhizobium meliloti* (19). Our strategy was to order type II strains by multilocus enzyme electrophoresis and then to characterize these bacteria phenotypically. Representative strains were chosen for total DNA and ribosomal DNA hybridization and for the determination of partial 16S rRNA gene sequences.

On the basis of the criteria analyzed, we propose a new species, *Rhizobium tropici*, which contains two subgroups that correspond to type IIA and type IIB strains.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1.

Growth conditions. Rhizobia were maintained on yeast extract-mannitol (YM) medium (65), on peptone-yeast extract (PY) medium, (43), or in minimal medium (MM) (17) containing different substrates. Average doubling times were estimated from optical densities recorded at 600 nm every 2 h in PY medium at 30°C. Bacterial swarming was tested by growing strains for 2 days on PY medium supplemented with 0.3% agar.

Nodulation and nitrogen fixation were tested in sterilized Leonard jars (65) containing vermiculite and sand by using *P. vulgaris* cv. Carioca 80 and *L. leucocephala*.

Multilocus enzyme electrophoresis. Cultures derived from single colonies were grown overnight at 30°C in 50 ml of PY

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TABLE 1. Bacterial strains and ETs

Strain	Original host plant	ET[a]	Source or reference[b]
Rhizobium leguminosarum biovar phaseoli (type I)			
CFN 42	*Phaseolus vulgaris* L.		46
Viking I	*Phaseolus vulgaris* L.		49
TAL 182	*Phaseolus vulgaris* L.		Ben Bohlool
BR 10027	*Phaseolus vulgaris* L.		CNPBS
BR 10028	*Phaseolus vulgaris* L.	36	CNPBS
BR 10029	*Phaseolus vulgaris* L.		CNPBS
BR 10030	*Phaseolus vulgaris* L.	37	CNPBS
Rhizobium leguminosarum biovar trifolii			
USDA 2046	*Trifolium pratense* L.		USDA
USDA 2152	*Trifolium subterraneum* L.		USDA
Rhizobium leguminosarum biovar viciae			
USDA 2489	*Vicia faba* L.		USDA
Rhizobium meliloti			
RCR 2011	*Medicago sativa*		51
R.me 1	*Medicago sativa*		CFN
Rhizobium fredii			
USDA 191	*Glycine max*		32
HH 103	*Glycine max*		16
Rhizobium galegae 625 (= gal 3)	*Galega officinalis*		66
Rhizobium loti NZP 2037	*Lotus divaricatus*	39	10
Rhizobium spp.			
CFN 234	*Leucaena leucocephala*	40	39
CFN 265	*Leucaena esculenta*		39
NGR 234		38	60
Type IIA			
CFN 299	*Phaseolus vulgaris* L.	1	38
BR 828	*Leucaena leucocephala*	1	CNPBS
BR 829	*Leucaena leucocephala*	1	CNPBS
BR 830	*Leucaena leucocephala*	1	CNPBS
BR 831	*Leucaena leucocephala*	1	CNPBS
BR 832	*Leucaena leucocephala*	1	CNPBS
BR 833	*Leucaena leucocephala*	1	CNPBS
BR 834	*Leucaena leucocephala*	1	CNPBS
BR 835	*Leucaena leucocephala*	1	CNPBS
BR 836	*Leucaena leucocephala*	1	CNPBS
BR 10031	*Phaseolus vulgaris* L.	1	CNPBS
BR 10032	*Phaseolus vulgaris* L.	1	CNPBS
BR 10033	*Phaseolus vulgaris* L.	1	CNPBS
BR 10034	*Phaseolus vulgaris* L.	1	CNPBS
BR 10035	*Phaseolus vulgaris* L.	1	CNPBS
BR 10036	*Phaseolus vulgaris* L.	1	CNPBS
BR 10037	*Phaseolus vulgaris* L.	1	CNPBS
BR 10038	*Phaseolus vulgaris* L.	1	CNPBS
BR 837	*Leucaena leucocephala*	1	CNPBS
BR 838	*Leucaena leucocephala*	1	CNPBS
BR 839	*Leucaena leucocephala*	1	CNPBS
BR 840	*Leucaena leucocephala*	1	CNPBS
BR 10039	*Phaseolus vulgaris* L.	1	CNPBS
BR 841	*Leucaena leucocephala*	1	CNPBS
C-05-1	*Phaseolus vulgaris* L.	1	Tsai
Car 22	*Phaseolus vulgaris* L.	1	Tsai
UMR 1163	*Phaseolus vulgaris* L.	1	Graham
BR 10040	*Phaseolus vulgaris* L	2	CNPBS
BR 10041	*Phaseolus vulgaris* L.	3	CNPBS
BR 10042	*Phaseolus vulgaris* L.	4	CNPBS
BR 842	*Leucaena leucocephala*	5	CNPBS
BR 843	*Leucaena leucocephala*	6	CNPBS
BR 844	*Leucaena leucocephala*	7	CNPBS
BR 845	*Leucaena leucocephala*	8	CNPBS
C-05-35	*Phaseolus vulgaris* L.	9	Tsai
BR 10043	*Phaseolus vulgaris* L.	10	CNPBS
BR 10044	*Phaseolus vulgaris* L.	11	CNPBS
BR 10045	*Phaseolus vulgaris* L.	12	CNPBS
BR 846	*Leucaena leucocephala*	13	CNPBS
UMR 1178 (= IAPAR 47)	*Phaseolus vulgaris* L.	14	Graham

Continued on following page

TABLE 1—*Continued*

Strain	Original host plant	ET[a]	Source or reference[b]
Type IIB			
BR 847	*Leucaena leucocephala*	15	CNPBS
BR 848	*Leucaena leucocephala*	15	CNPBS
BR 849	*Leucaena leucocephala*	15	CNPBS
BR 850	*Leucaena leucocephala*	16	CNPBS
BR 851	*Leucaena leucocephala*	17	CNPBS
BR 852	*Leucaena leucocephala*	18	CNPBS
BR 853	*Leucaena leucocephala*	19	CNPBS
BR 854	*Leucaena leucocephala*	20	CNPBS
UMR 1410 (= CIAT 166)	*Phaseolus vulgaris* L.	21	Graham
CIAT 899[T]	*Phaseolus vulgaris* L.	22	25
AD 822		22	Quinto
AD 4		22	Quinto
BR 855	*Leucaena leucocephala*	23	CNPBS
BR 856	*Leucaena leucocephala*	24	CNPBS
C-05 II	*Phaseolus vulgaris* L.	24	Tsai
BR 857	*Leucaena leucocephala*	25	CNPBS
BR 858	*Leucaena leucocephala*	26	CNPBS
BR 859	*Leucaena leucocephala*	27	CNPBS
BR 860	*Leucaena leucocephala*	28	CNPBS
BR 861	*Leucaena leucocephala*	29	CNPBS
BR 862	*Leucaena leucocephala*	30	CNPBS
CFNE 101	*Phaseolus vulgaris* L.	31	CFN
BR 863	*Leucaena leucocephala*	32	CNPBS
Type II			
UMR 1226 (= IAPAR 70)	*Phaseolus vulgaris* L.	33	Graham
BR 864	*Leucaena leucocephala*	34	CNPBS
UMR 1173 (IAPAR 69)	*Phaseolus vulgaris* L.	35	Graham
Agrobacterium tumefaciens C58			61

[a] ET is the combination of mobility alleles of electromorphs.

[b] Sources: Ben Bohlool, B. Ben Bohlool, NifTAL Project, Paia, Hawaii; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico; CNPBS, Centro Nacional de Pesquisa em Biología do Solo, Seropédica 23851, Rio de Janeiro, Brazil; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, Md.; Graham, P. Graham, Department of Soil Sciences, University of Minnesota, St. Paul; Tsai, M. Tsai, Universidade de Sao Paulo, Sao Paulo, Brazil; Quinto, C. Quinto, Centro de Ingeniería Genética y Biotecnología, Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico.

medium and then centrifuged, suspended in 1 ml of 10 mM $MgSO_4$, and sonicated twice for 20 s with a 20-s rest by using an MSE sonifier equipped with a microtip at 50% pulse with ice cooling. Lysates were stored at −70°C.

The procedures used for starch gel electrophoresis and activity assays for specific enzymes have been described by Selander et al. (56). The following eight metabolic enzymes were assayed: alcohol dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, xanthine dehydrogenase, indophenol oxidase (superoxide dismutase), hexokinase, and phosphoglucomutase. The buffer system used was Tris-citrate (pH 8). The mobility variants of each enzyme were numbered in order of decreasing anodal mobility. At least five different electrophoretic assays were performed for each of the 65 strains for each enzyme tested. The distinctive combinations of electromorphs (mobility variants of each enzyme) were designated electrophoretic types (ETs) (56). The ET was determined for each strain.

The genetic diversity for an enzyme locus was calculated as follows: $h = (1 - \Sigma x_i^2)n/(n - 1)$, where x_i^2 is the frequency of the ith allele and n is the number of ETs. The mean genetic diversity per locus (H) was the arithmetic average of h values for the eight loci (56). The genetic distance between each pair of ETs was estimated as the proportion of loci at which dissimilar alleles occurred. Clustering from a matrix of pairwise genetic distances was performed by using the average linkage method (58).

DNA-DNA hybridization. DNA was purified from cells that were treated with sodium dodecyl sulfate (1%, wt/vol), Pronase (50 μg/ml), and RNase (10 μg/ml) and then subjected to serial extractions with phenol-chloroform (1:1, vol/vol) and precipitation with NaCl and ethanol. The DNA concentration was estimated spectrophotometrically at 260 nm. Total DNA digested with *Eco*RI was subjected to electrophoresis in 1% agarose gels. The DNA was transferred to nylon filters (59) and hybridized (21) to DNA previously digested with *Eco*RI and labeled with ^{32}P by nick translation (48) (10^8 cpm/μg of DNA). The labeled DNAs were from three reference strains, strains CFN 299 (type IIA), CIAT 899[T] (T = type strain) (type IIB), and *Rhizobium meliloti* RCR 2011. Autoradiography was performed at −70°C for 24 h; filter lanes were cut and counted with a Beckman scintillation counter. The percentage of total homologous hybridization was calculated for each strain tested.

Ribosomal DNA hybridization. The restriction fragment length polymorphisms of the rRNA operons were determined by hybridizing total DNA *Eco*RI, *Xho*I, and *Hin*dIII digests probed with plasmid pKK3535 (7). This plasmid carries a 7.5-kb *Bam*HI fragment containing the *Escherichia coli rrnB* operon cloned in plasmid pBR322.

Numerical taxonomy. A total of 51 strains were characterized, and 118 different characteristics were analyzed. For testing substrate utilization, 5-μl drops of freshly prepared bacterial suspensions (approximately 10^5 bacteria) were ap-

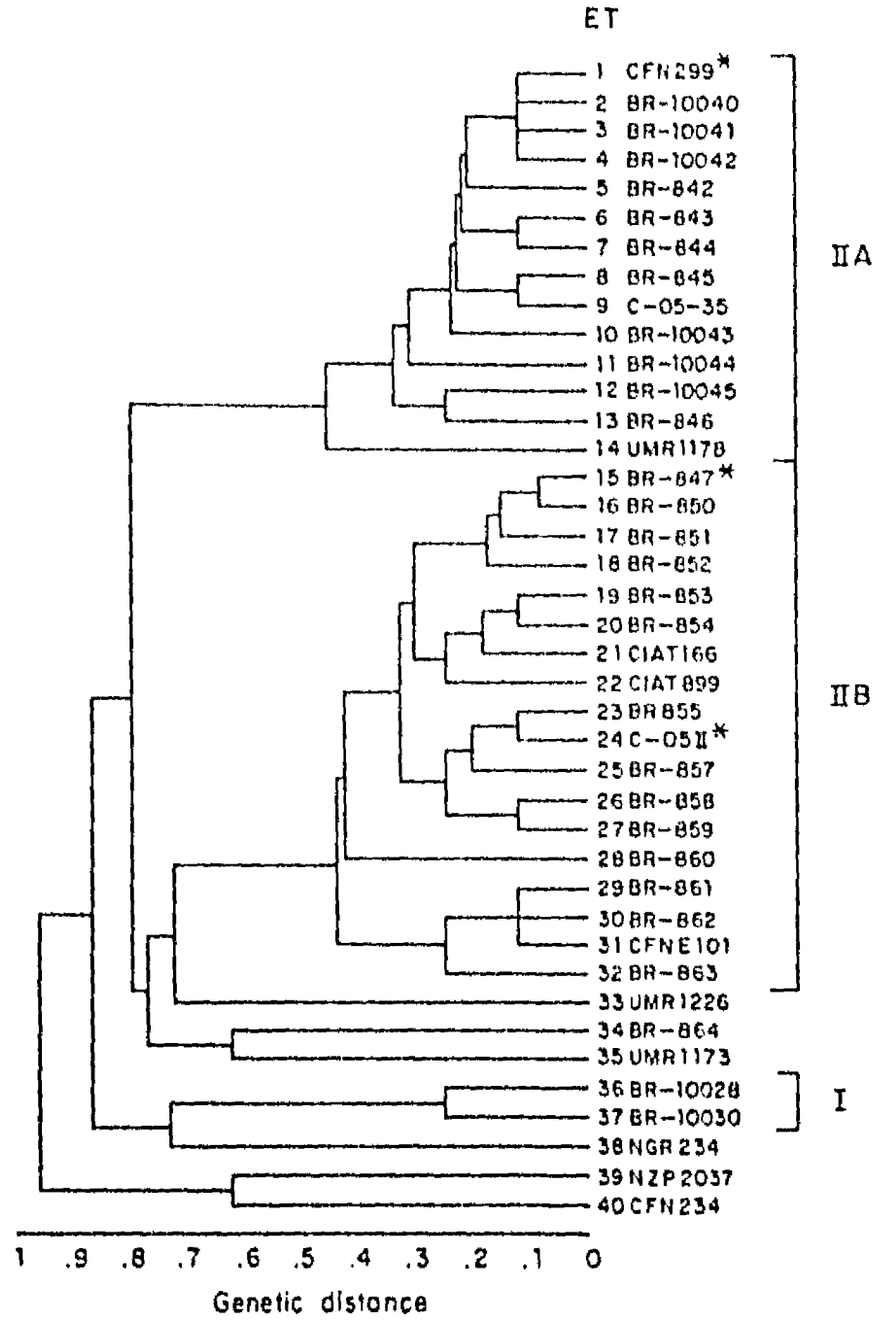

FIG. 1. Dendrogram showing levels of genetic relatedness among 35 ETs of type II strains, 2 ETs of type I strains, and 3 ETs of outgroup reference strains. This dendrogram was based on electrophoretically detectable allelic variation at enzyme loci. The asterisks indicate that other strains having the same ET are included in Table 1.

TABLE 2. Allele profiles at eight enzyme loci of 40 ETs

ET	Type or taxon	No. of isolates	Alleles at the following enzyme loci[a]:							
			HEX	IDH	XDH	MDH	ADH	IPO	G6P	PGM
1	IIA	27	5	5	5	5	5	5	5	5
2	IIA	1	5	5	5	5	5	5	5	5.1
3	IIA	1	5	5	5	5	5	5	5	4.9
4	IIA	1	5	5	5	5	5	5	5	5.5
5	IIA	1	5	5	5	5.5	5	5	5	5
6	IIA	1	5	5	4.8	5	5	5	5	5
7	IIA	1	5	5	5.5	5	5	5	5	5
8	IIA	1	5	4.5	5	5	5	5	5	5
9	IIA	1	5	6	5	5	5	5	5	5
10	IIA	1	4.9	5	5	5	5	5	5	5
11	IIA	1	5.5	5	5	5	5	5	5	6
12	IIA	1	5	4.8	5.1	5	5	5	5	5
13	IIA	1	5	5	5.1	5	6	5	5	5
14	IIA	1	5	5	5.5	5	4.7	5	5.2	5
15	IIB	3	5	4	6	6	4	7	4	5
16	IIB	1	5	4	6	6	5.9	7	4	5
17	IIB	1	5	4	6	6	4	6.5	4	5
18	IIB	1	5	3.5	6	6	4	7	4	5
19	IIB	1	5	4	6.1	6	4	7	4	5
20	IIB	1	5.5	4	6.1	6	4	7	4	5
21	IIB	1	5	4.5	6.1	6	4	7	4	5
22	IIB	1	4.5	3	6.1	6	4	7	4	5
23	IIB	1	5.2	5	6.1	6	4	7	4	5
24	IIB	2	5.2	5	6	6	4	7	4	5
25	IIB	1	5.2	3.9	6.1	6	4	7	4	5
26	IIB	1	5.2	4	6	6	4	7	5	5
27	IIB	1	5.2	4	6	6	4	7	4	5
28	IIB	1	5	5	4.5	6	5.8	7	4	5
29	IIB	1	5	4	7	6	4.5	5	4	5
30	IIB	1	5	4	6.1	6	4.5	5	4	5
31	IIB	1	5	4	6	6	4.5	5	4	5
32	IIB	1	5	4	5	6	4.5	7	4	5
33	II	1	7	6	6.1	4.9	4	5	7	5
34	II	1	5	4.5	6	6	5.9	5	6	4
35	II	1	6	6	4.5	6	5.8	5	6	5.5
36	I	1	5	3	4	4	6	6	7	5.5
37	I	1	3	3	4	4	6	6	7	4
38	*Rhizobium loti*	1	2	3	1	1		2	4.9	5
39	*Rhizobium* sp.	1	6	6	5	4	7	8	6	6
40	*Rhizobium* sp.	1	6	6	7	3	3	4	5.5	6

[a] HEX, hexokinase; IDH, isocitrate dehydrogenase; XDH, xanthine dehydrogenase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; IPO, indophenol oxidase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase.

plied to plates containing MM (17) lacking vitamins to which filter-sterilized substrates had been added. When substrates were tested as nitrogen sources, ammonium sulfate was not included and glucose was added at a concentration of 1 g/liter. The plates were incubated at 30°C unless indicated otherwise. The following compounds were tested for utilization as sole carbon sources (at a concentration of 1 g/liter unless indicated otherwise): L-alanine, L-arginine, L-aspartate, L-phenylanine, glycine, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-lysine, L-histidine, L-methionine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan, L-valine, hypoxanthine, ornithine, nopaline, octopine, α-ketoglutarate, D-fructose, D-galactose, D-glucose, D-glucosamine, D-glucose-6-phosphate, lactose, D-glucuronate, D-mannose, mannitol, D-ribose, sorbose, D-sorbitol, succinate, acetate, anthranilate, casein hydrolysate, citrate, formate, isovalerate, D-malate, nicotinate, oxalate, L-tartrate, starch, sarcosine, urea, glycerol, ethanol, phenol (0.25 g/liter), and methanol. The following compounds were tested for utilization as sole nitrogen sources (at a concentration of 0.5 g/liter): ammonium sulfate, L-aspartate, glycine, L-glutamate, L-glutamine, ornithine, L-tyrosine, and L-tryptophan. We also determined requirements for ascorbic acid (100 μg/ml), biotin (100 μg/ml), folic acid (100 μg/ml), and pantothenate (100 μg/ml).

Tolerance to antibiotics and tolerance to sodium hypochloride were tested by growing organisms on MM containing kasugamycin, lincomycin, oleandomycin, sulfamide, or trimethoprim (each at a concentration of 20 μg/ml) or by growing organisms on PY medium containing carbenicillin (30 or 50 μg/ml), chloramphenicol (30 or 100 μg/ml), erythromycin (100 μg/ml), gentamicin (25 μg/ml), kanamycin (30 μg/ml), neomycin (60 μg/ml), novobiocin (20 μg/ml) polymyxin B (20 μg/ml), rifampin (50 μg/ml), spectinomycin (100 μg/ml), streptomycin (100 μg/ml), tetracycline (1, 5, or 10 μg/ml), or sodium hypochloride (0.12%, wt/vol).

Additional tests included growth on PY medium at 10, 30, 37, and 40°C; growth on PY medium containing 1.0, 1.5, and 2% NaCl; growth on PY medium at pH 4, 5, 6, 8.5, and 10.5; growth on liquid PY medium lacking calcium; growth on

TABLE 3. Genetic diversity at eight enzyme loci among ETs

Enzyme locus[a]	Characteristics of:							
	40 ETs[b]		35 ETs[c]		18 ETs[d]		14 ETs[e]	
	No. of alleles	h[f]	No. of alleles	h	No. of alleles	h	No. of alleles	h
HEX	9	0.615	7	0.537	4	0.576	3	0.275
IDH	8	0.785	8	0.737	6	0.621	4	0.396
XDH	10	0.494	8	0.808	5	0.680	4	0.582
MDH	7	0.646	4	0.543	1	0	2	0.142
ADH	9	0.806	7	0.739	4	0.530	3	0.275
IPO	7	0.641	3	0.532	3	0.386	1	0
G6P	7	0.696	5	0.611	2	0.111	2	0.142
PGM	7	0.391	7	0.309	1	0	5	0.506
Mean	8	0.619	6	0.602	3	0.363	3	0.289

[a] For abbreviations see Table 2, footnote *a*.
[b] The total sample for 40 ETs examined.
[c] The 35 ETs of the *Rhizobium leguminosarum* biovar phaseoli type II strains.
[d] The 18 ETs of the type IIB strains.
[e] The 14 ETs of the type IIA strains.
[f] $h = (1 - \Sigma x_i^2)\, n/(n-1)$, where x_i^2 is the frequency of the *i*th allele and *n* is the number of ETs.

Luria broth (LB); colony morphology on PY medium, YM medium, and MM containing various carbon sources; and acid production on YM medium containing bromothymol blue (0.0025%, wt/vol) as an indicator. Plates were incubated at 30°C unless otherwise specified, and growth was recorded at 3 and 5 days after inoculation. The results were analyzed by the mixed parsimony method, using the Wagner criterion (33).

Nucleotide sequences of 16S rRNA genes. The nucleotide sequences of the 16S rRNA genes of type I strain CFN 42 and type II strains CIAT 899^T, CFN 299, and UMR 1173 were determined by directly sequencing double-stranded polymerase chain reaction products with Sequenase 2 (U.S. Biochemical Corp.). A 491-bp region corresponding to nucleotides 872 through 1,363 of the *A. tumefaciens* 16S rRNA gene was amplified by using a GenAmp DNA amplification reagent kit (Perkin Elmer Cetus) with a 28-mer (CCGCA CAAGCGGTGGAGCATGTGGTTTA) and a 30-mer (CTTG TACACACCGCCCGTCACACCATGGGA) as primers. The reaction was carried out according to the instructions of the manufacturer by using 30 cycles, as follows: 30 s at 95°C for denaturation, 30 s at 55°C for primer annealing, and 3 min for polymerization at 72°C. The polymerase chain reaction products were purified by using QIAGEN tip 20 minicolumns as recommended by the manufacturer.

Both strands of three independent double-stranded polymerase chain reaction products from each strain tested were sequenced with Sequenase by using the method of Casanova et al. (8) and the same primers as those used in the amplification procedure.

We used the program LINEUP to manually align the sequences with the following corresponding sequences obtained from GenBank: *Rickettsia rickettsii* M21293, *Rickettsia typhi* M20499, *Rickettsia prowazekii* M21789, *A. tumefaciens* M11223, *Rochalimea quintana* M11927, and *Brucella abortus* X13695. Phylogenetic distances were determined by using the DISTANCES program of the University of Wisconsin GCG Sequence Analysis Software Package (14). An unweighted pair group method tree was constructed, with the standard errors of branch points determined by using the unweighted pair group method standard error program (42).

TABLE 4. Relative levels of homology at 65°C between DNAs from *Rhizobium* species and reference DNAs from type IIA, type IIB, and *Rhizobium meliloti* strains

Strain	% Of DNA hybridization with the following reference strains:		
	CFN 299 (type IIA)	CIAT 899^T (type IIB)	*R. meliloti* RCR 2011[a]
Rhizobium leguminosarum biovar phaseoli			
Viking I	21	15	
CFN 42	26	19	
Rhizobium leguminosarum biovar trifolii			
USDA 2046	20	30	
USDA 2152	17	23	
Rhizobium leguminosarum biovar viciae USDA 2489	16	27	
Rhizobium meliloti RCR 2011	20	15	100
Rhizobium fredii			
USDA 191	15	24	20
HH 103	20	23	
Rhizobium galegae 625	12	24	25
Rhizobium loti NZP 2037	10	15	19
Rhizobium spp.			
CFN 234	21	38	
CFN 265	16	27	
NGR 234		15	
Type IIA			
CFN 299	100	36	29
C-05-35	96	26	22
UMR 1178	73	14	
BR 10035	98		
Type IIB			
CIAT 899^T	39	100	14
UMR 1410		88	
BR 859		72	
BR 856		85	
BR 863		62	
Type II			
BR 864	18	22	
UMR 1173		38	
UMR 1226		28	
Agrobacterium tumefaciens C58	10	17	

[a] *Rhizobium meliloti* RCR 2011 was included only as a reference strain to test the hybridization conditions used in this work.

Nucleotide sequence accession numbers. The ribosomal gene sequences reported below for the different strains have been deposited in GenBank/EMBL nucleotide sequence databases under accession numbers M64317, M64318, M64319, and M64405.

RESULTS

Multilocus enzyme electrophoresis. Figure 1 shows that the type II strains were divided into two groups (types IIA and IIB), both of which differed from type I strains. Type II strains and type I strains were at a genetic distance of 0.86, while type IIA strains and type IIB strains were at a genetic distance of 0.79. Type IIA strains exhibited greater homogeneity than type IIB strains; the mean genetic diversity was 0.289 for the former and 0.363 for the latter. A total of 27 type IIA strains from various geographical origins were identical as determined by the mobilities of the eight metabolic enzymes tested and formed ET 1. The majority of the bean isolates tested could be separated into three groups on

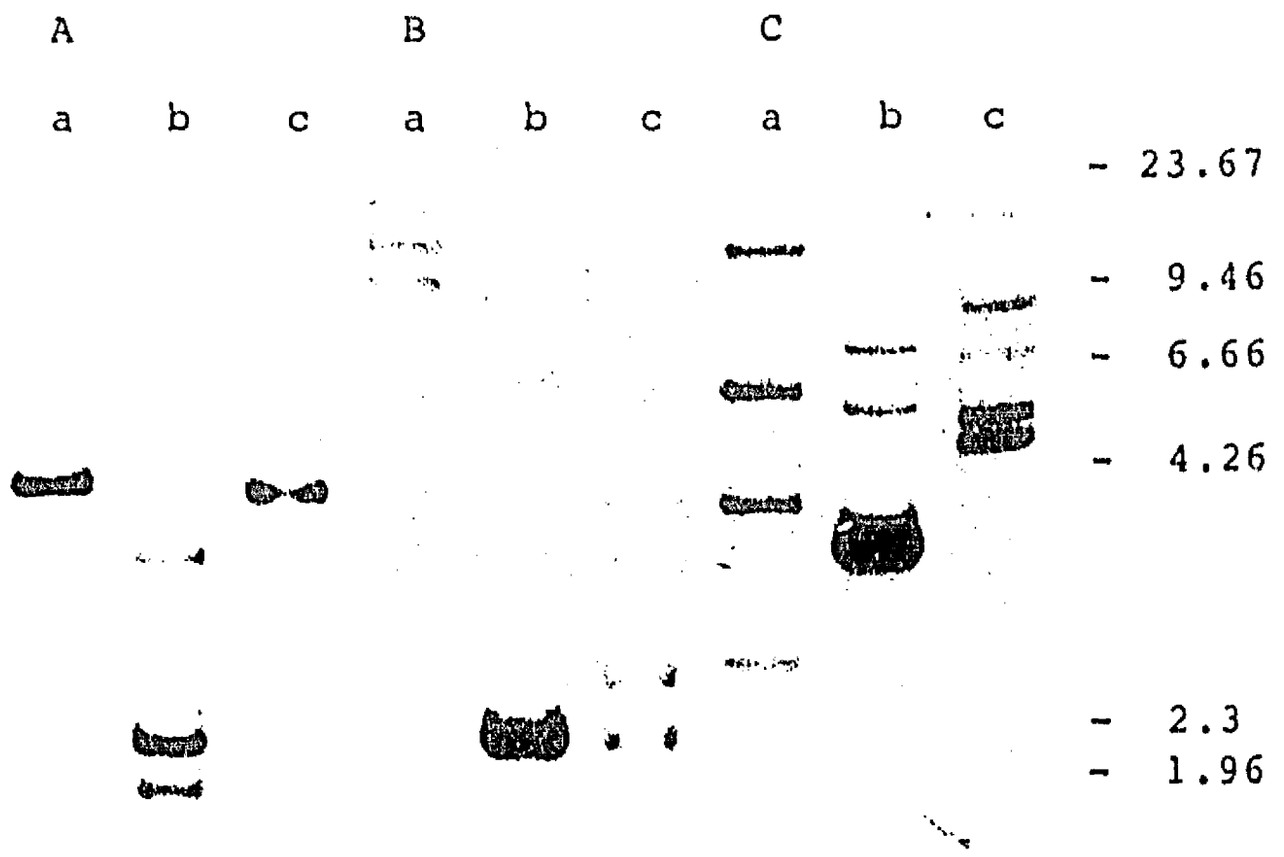

FIG. 2. Autoradiogram of *Eco*RI (A), B) *Hin*dIII (B), and *Xho*I (C) ribosomal restriction fragment length polymorphism patterns of type I strain CFN 42 (lanes a), type IIA strain CFN 299 (lanes b), and type IIB strain CIAT 899^T (lanes c). The positions of the molecular weight markers (in kilobases) are shown on the right.

the basis of the allelic responses at the loci for malate dehydrogenase and, in the majority of the strains, at the loci for indophenol oxidase (Table 2); these groups basically corresponded to type I, type IIA, and IIB type strains. Type IIA and IIB strains shared alleles at the hexokinase and phosphoglucomutase loci, but exhibited very small mobility differences at the glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and xanthine dehydrogenase loci. Alcohol dehydrogenase activity was difficult to detect in type IIB strains but not in type IIA strains. The genetic diversity at each enzyme locus is shown in Table 3.

To determine the location of the genes coding for these metabolic enzymes in type II strains, derivatives of strains CIAT 899^T, AD 4, and AD 822 lacking either the 200-kb plasmid or the 400-kb plasmid were evaluated. Identical enzyme mobility variants were obtained for all eight enzymes tested, suggesting that, as in *E. coli* (56), these traits are chromosomally determined.

ETs 33, 34, and 35 shared some phenotypic characteristics with type IIB strains but were separated from them by a genetic distance of 0.78, low levels of DNA-DNA hybridization with type IIB reference strain CIAT 899^T (Table 4), and differences in ribosomal gene sequences (see below).

DNA-DNA hybridization. Four type IIA strains and five type IIB strains constituted homogeneous groups with relatively high levels of DNA homology (91.7% for type IIA strains with reference strain CFN 299 and 81.4% for type IIB strains with reference strain CIAT 899^T) (Table 4). DNAs from other *Rhizobium* species, including *Rhizobium leguminosarum* biovar phaseoli, *Rhizobium leguminosarum* biovar trifolii, and *Rhizobium leguminosarum* biovar viciae (27), as well as *Rhizobium galegae* (35, 66), *Rhizobium loti* (28), *Rhizobium meliloti*, *Rhizobium fredii* (9, 53), and unclassified rhizobia, exhibited less than 30% hybridization with total DNA from either strain CFN 299 or strain CIAT 899^T.

Ribosomal gene organization and sequence. Figure 2 shows the restriction fragment length polymorphisms of rRNA operons of strains CFN 42 (type I), CFN 299 (type IIA), and CIAT 899^T (type IIB); the hybridization patterns for these strains were clearly different. Four type IIA strains had patterns identical to the pattern of strain CFN 299 in *Eco*RI digests. Similarly, seven type IIB strains had the same restriction fragment length polymorphisms in *Eco*RI digests as strain CIAT 899^T (data not shown).

Figure 3 shows the DNA sequences of the 16S RNA gene fragments obtained from strains CFN 42 (type I), CFN 299 (type IIA), CIAT 899^T (type IIB), and UMR 1173 (type II, ET 35), and Fig. 4 shows the phylogenetic tree obtained by

```
          5'--->3'
          1                                                    50
          CtGtAGAGat gCAGGGtcaC TTCGGtggCG aagaACAGGT GCTGCATGGC
Cfn42      T C    GC A     GTT      GAC   GCAC
Cfn299     A C    AG T     TCA      TGG   AAGA
Ciat899    T T    AT G     TCA      TGG   AAGA
Umr1173    T T    AT G     TCA      TGG   AAGA

          51                                                  100
          TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC
          101                                                 150
          AACCCTCGCC CTTAGTTGCC AGCATTtaGT TGGGCACTCT AAGGGGACTG
Cfn42                                TG
Cfn299                               TA
Ciat899                              CA
Umr1173                              TA

          151                                                 200
          CCGGTGATAA GCCGAGAGGA AGGTGGGGAT GACGTCAAGT CCTCATGGCC
          201                                                 250
          CTTACGGGCT GGGCTACACA CGTGCTACAA TGGTGGTGAC AGTGGGCAGC
          251                                                 300
          GAGCACGCGA GTGTGAGCTA ATCTCCAAAA GCCATCTCAG TTCGGATTGC
          301                                                 350
          ACTCTGCAAC TCGAGTGCAT GAAGTTGGAA TCGCTAGTAA TCGCGGATCA
          351
          GCATGC
```

FIG. 3. Aligned sequences of parts of the 16S rRNA genes from strains CFN 42 (type I), CFN 299 (type IIA), CIAT 899^T (type IIB), and UMR 1173 (type II), corresponding to nucleotides 954 to 1,109 from the *A. tumefaciens* gene. Only the differences from the consensus sequence (at the top) are shown. From nucleotide 151 on the four sequences are identical.

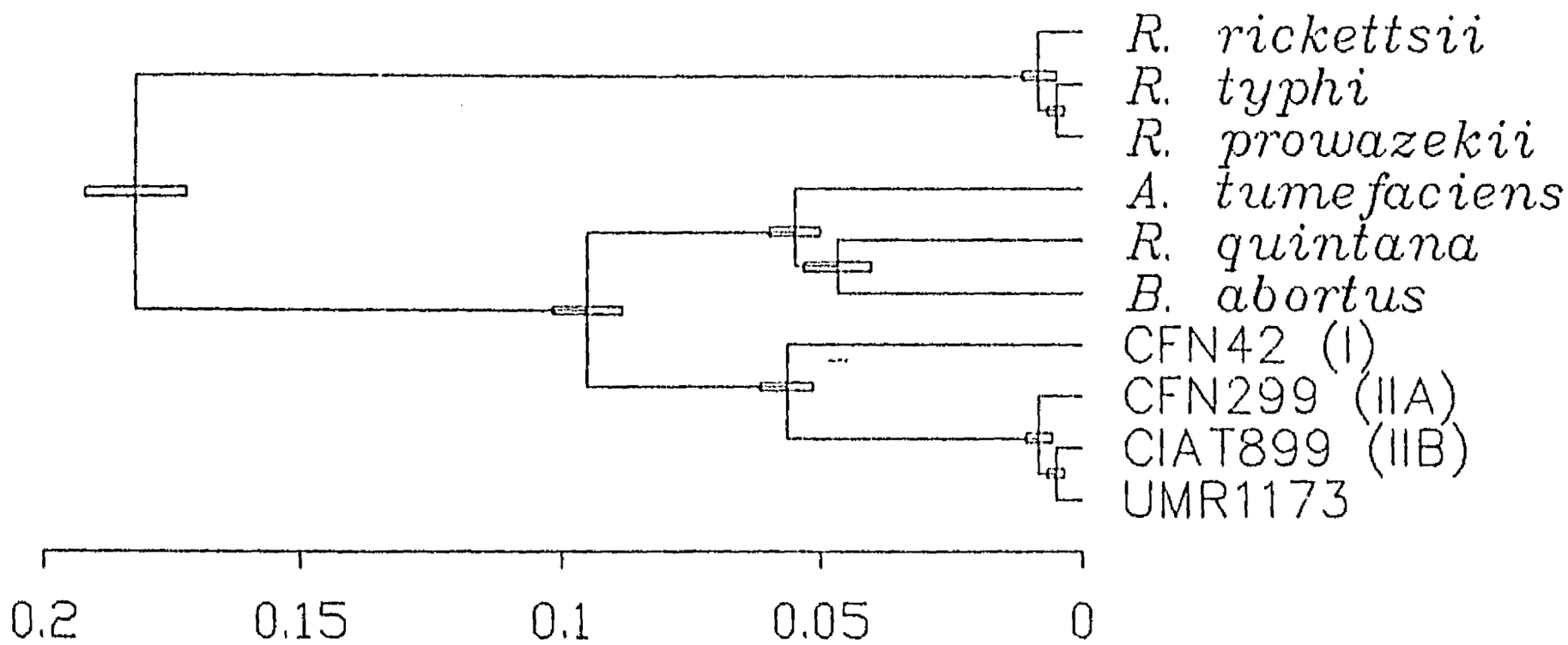

FIG. 4. Unweighted pair group with branching point standard error tree (42) derived from 16S RNA gene fragment sequences of *Rickettsia rickettsii*, *Rickettsia typhi*, *Rickettsia prowazekii*, *A. tumefaciens*, *Rochalimea quintana*, *Brucella abortus*, *Rhizobium leguminosarum* biovar phaseoli, and type II strains.

the unweighted pair group method. The tree is in agreement with the known phylogeny of proteobacteria. The three type II strains formed independent branches that were separated from type I strain CFN 42, and these strains formed a different cluster than the other members of the *Rhizobiaceae*, which in turn were in a different lineage than the rickettsiae. The internal phylogeny of type II strains is not clearly defined, as shown by the overlapping of the standard error bars in Fig. 4.

Numerical taxonomy. We characterized 51 strains, 35 type II strains representing each of the ETs of type II strains and 16 other strains, including *Rhizobium leguminosarum* biovar phaseoli, *Rhizobium leguminosarum* biovar trifolii, *Rhizobium leguminosarum* biovar viciae, *Rhizobium meliloti*, *Rhizobium galegae*, *Rhizobium loti*, and *Rhizobium* sp. strain NGR 234.

The rhizobia did not utilize the following compounds as carbon sources: starch, nicotinate, oxalate, ethanol, methanol, phenol, L-methionine, L-phenylalanine, L-threonine, L-alanine, and L-valine. No strain grew on PY medium at pH 3 or 4 or on PY medium supplemented with 1, 1.5, or 2% NaCl. All of the strains tested grew on α-ketoglutarate, D-fructose, D-galactose, D-glucose, D-glucosamine, glucuronate, D-mannose, mannitol, D-ribose, L-tyrosine, and L-tryptophan as carbon sources and on L-glutamate, L-glutamine, and L-tyrosine as nitrogen sources. Table 5 shows some of the relevant phenotypic characteristics of the strains. The results of the complete-linkage cluster analysis obtained by the mixed parsimony method in which 118 characteristics were considered are shown in Fig. 5; these results are in agreement with the dendrogram derived from multilocus enzyme electrophoresis, but on the basis of the phenotypic characteristics the type IIA and type IIB clusters appear to be more distinct.

TABLE 5. Relevant phenotypic characteristics of *Rhizobium* strains[a]

Characteristic	*Rhizobium leguminosarum* biovar phaseoli	Type IIA strains	Type II strains
Nodulation and nitrogen fixation in *Leucaena* spp.	−	+	+
Colony morphology on PY medium	Gummy	Creamy	Creamy
Growth on LB	−	−	+
Growth on PY medium lacking calcium	−	−[b]	+[c]
Growth on PY medium containing antibiotics[d]	−	−	+[c]
Growth on MM containing arginine as a C source	−	−	+
Growth on MM containing malate	+[c]	−[b]	+[c]
Growth on MM containing hypoxanthine	−[f]	−	+[c]
Growth on MM containing sorbitol	−[f]	−	+[c]
Maximum growth temp (°C)	35	37	40
Colony morphology on YM medium	Wet, translucent	White, opaque	Wet, translucent
Motility on 0.3% agar	+	−	+

[a] The substrate and antibiotic concentrations used are described in Materials and Methods.
[b] More than 90% of the strains were negative.
[c] More than 90% of the strains were positive.
[d] The antibiotic used was carbenicillin, spectinomycin, chloramphenicol, or rifampin.
[e] More than 60% of the strains were positive.
[f] More than 60% of the strains were negative.

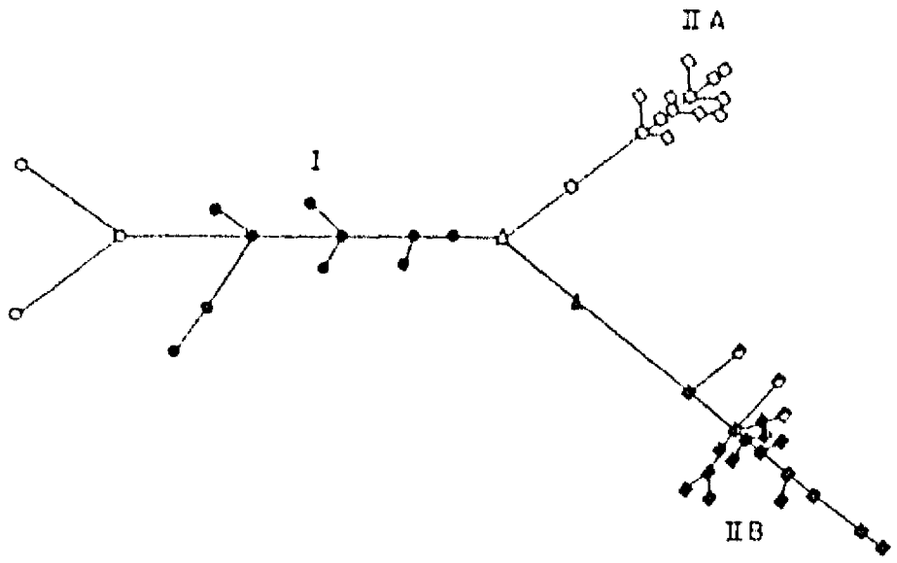

FIG. 5. Cladogram derived from a mixed parsimony analysis of the phenotypic characteristics of *Rhizobium meliloti* and strain NGR 234 (○), *Rhizobium leguminosarum* (●), *Rhizobium loti* (△), and *Rhizobium galegae* (▲), as well as type IIA strains (◇), type IIB strains (◆), and type II unclassified strains (ET 33, 34, and 35) (◈).

General characteristics. Type II strains are gram-negative, rod-shaped, nonsporeforming bacteria that are 1.5 to 2 μm long, are peritrichous, and produce acid in YM medium. The average doubling times are 2 and 1.67 h for type IIA and type IIB strains, respectively, at 30°C in PY medium. These organisms do not produce 3-ketolactose (1) but do grow in MM containing lactose, and they are nalidixic acid resistant, as are most *Rhizobium leguminosarum* biovar phaseoli strains. The type II strains listed in Table 1 nodulate *P. vulgaris* cv. Carioca 80, and some strains are as efficient as the best type I strains.

Type IIA strains are nonmotile on soft agar, while type I and IIB strains are motile. Only about 10% of the 64 type II strains analyzed produce melanin, whereas this is a very a common characteristic among *Rhizobium leguminosarum* biovar phaseoli strains (4).

DISCUSSION

Research on rhizobia that nodulate bean plants (*P. vulgaris*) has frequently revealed strains which have behavior that is considered atypical for *Rhizobium leguminosarum* (5, 23, 25, 26, 38, 39, 64, 70). Nevertheless, all of these organisms have been classified as *Rhizobium leguminosarum* biovar phaseoli, which has resulted in a genetically heterogeneous group. Therefore, we propose that a group of these strains should be assigned to a new species, *Rhizobium tropici*. The considerations described below support such an assignment. These bacteria have a wider host range, including *Leucaena* spp., carry single *nif* gene copies, and exhibit low levels of DNA-DNA hybridization with other *Rhizobium* species. Furthermore, the genetic distances as calculated by multilocus enzyme electrophoresis and by 16S rRNA sequence comparisons are well beyond the acceptable threshold that separates bacterial species.

Like other *Rhizobium* species (13, 22), *Rhizobium tropici* sp. nov. strains have two glutamine synthetases (20), and the *nod* and *nif* genes are plasmid borne (5, 38). Our results for the general pattern of utilization of carbon compounds are in accordance with the patterns reported by Dreyfus et al. for *Rhizobium* strains (17). Melanin production was not considered as a phenotypic characteristic in our taxonomic analysis as it is plasmid encoded in *Rhizobium leguminosarum* biovar phaseoli (4) and is widespread among different *Rhizobium* species (11).

Rhizobium leguminosarum biovar phaseoli (type I) strains have been reported to be an assembly of lineages with considerable genetic distances among them (45). *Rhizobium tropici* sp. nov. also encompasses at least two distinct clusters. Strains belonging to one of the groups (type IIA) require calcium for growth on PY medium and do not grow on LB. They form white opaque colonies on YM medium and are nonmotile on 0.3% agar. The maximum temperature for growth is 35 to 37°C. However, type IIB strains do not require calcium on PY medium, do grow on LB, form wet translucent colonies on YM medium, and are motile on 0.3% agar, and their maximum temperature for growth is 40°C. In contrast to type IIA strains, type IIB isolates grow on arginine, malate, hypoxanthine, and sorbitol as carbon sources. They are resistant to chloramphenicol, carbenicillin, spectinomycin, rifampin, and the metals Ni, Pb, Co, Cu, Ag, and Cr (41a). Type IIA strains are susceptible to both the antibiotics and the metals. Taking into consideration these differences, taxonomists in the future may consider it convenient to define the two groups as subspecies.

Isolation of bacteria from *P. vulgaris* nodules does not always provide *Rhizobium leguminosarum* biovar phaseoli (15) or *Rhizobium tropici* sp. nov. strains. Under laboratory conditions, beans nodulate with a wide range of rhizobia (6, 18, 24, 34, 39, 52), in many cases effectively (39). A comprehensive taxonomy of these strains will require further research. ETs 33, 34, and 35 described above did not cluster with type I, type IIA, or type IIB strains, nor did FL strains obtained from nodules of bean plants grown in *Leucaena* fields (39, 45), bean rhizobium isolates from France (source, N. Amarger, INRA, 21034 Dijon Cedex, France), or strain B599 (from E. Schmidt, University of Minnesota, St. Paul) (52a). A high level of diversity among the tree rhizobia has been reported as well (71). Furthermore, *Rhizobium* taxonomy must deal with a large number of diverging lineages that share symbiotic capabilities (40). Additional genera and species of root and stem nodule bacteria will be needed to accommodate this diversity (69).

Description of *Rhizobium tropici* sp. nov. *Rhizobium tropici* (tro' pi. ci. Gr. n. *tropikos*, tropics; N. L. gen. n. *tropici*, from the tropics). These bacteria are aerobic, gram-negative, nonsporeforming flagellated rods that are 0.5 to 0.7 by 1.5 to 2 μm. Colonies are circular, convex, semitranslucent, and usually 2 to 4 mm in diameter within 2 to 4 days on PY agar medium. They grow on YM medium and PY medium, and some strains grow on LB. The optimum pH for growth ranges from 5 to 7, and the temperature at which growth occurs may be as high as 40°C. All strains are nalidixic acid resistant. These strains, which have been isolated from tropical areas, nodulate and fix nitrogen on *P. vulgaris*, *Leucaena esculenta*, and *Leucaena leucocephala*. They are distinguished from other species at the molecular level by the results of whole-DNA hybridization tests, their multilocus enzyme electrophoresis profiles, and their ribosomal gene sequences.

The well-studied type IIB strain CIAT 899 (= ATCC 49672) is designated the type strain. It has the characteristics described above for *Rhizobium tropici* sp. nov. Like other type IIB strains, it grows on LB, and it is resistant to heavy metals and to the antibiotics chloramphenicol, spectinomycin, carbenicillin, and streptomycin.

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ADDENDUM

At the request of our colleagues we classified other strains obtained from *P. vulgaris* nodules as follows: *Rhizobium leguminosarum* biovar phaseoli CIAT 151, CIAT 632, CIAT 652, CIAT 7123, CIAT 7033, CIAT 7100, CIAT 7116, CIAT 7047, CIAT 7052, CIAT 7061, CIAT 7062, CIAT 7064, CIAT 7070, Kim 5 Sm, H2C, Arg 641.2, Arg 634.2, Arg 634.1, Arg 645.1, Arg 637.2, Arg 651.2, Arg 629.2, Arg 640.2, Arg 632.2, Arg 648.1, Arg 651.1, Arg 646.1, and Arg 645.2; and *R. tropici* CIAT 7069, CIAT 2560, Arg 635.2, G348, G522, G763, G842, G867, and G887.

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Rhizobium etli sp. nov. is a branch of American rhizobia with different specificities.

Running Title: Rhizobia related to *R. etli* sp. nov.

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ABSTRACT

The phylogenetic analysis of American rhizobia from tropical legumes was performed by determing the nucleotide sequences of partial fragments of 16S rRNA genes. By these criteria, *R.* spp. are relatives to *R. etli*. *R.* spp. are broad-host range bacteria and *R. etli* sp. nov. is probably the species that coevolved with *Phaseolus vulgaris* bean. *R.* spp. differed from *R. etli* in many plasmid-borne traits. Nod-metabolites produced by *R.* spp. were analyzed. They were different from those produced by *R. etli*, as they were sulfated-labeled oligosaccharides. The study of clusters of genetically related bacteria may help to understand the evolution of specificity in *Rhizobium* spp.

DNA techniques such as DNA-DNA hybridization, nucleic acid fingerprinting and sequencing have deepened our understanding of microbial diversity. The renewed interest in bacterial taxonomy is due largely to these new approaches. At present, the analysis of ribosomal RNA gene sequences is the most reliable approach to define bacterial phylogenetic relationships (46). Phylogenies in the Rhizobiaceae family are being defined based on 16S rRNA gene sequencing (47,48,49) and other modern criteria (40). Members of this family belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* establish a complex symbiotic relationship with legume plants.

Phaseolus vulgaris bean plants establish symbiosis with *R. etli* sp. nov. (36), formerly considered as *R. leguminosarum* bv. phaseoli, type I, *R. tropici* (22), and with other tropical *Rhizobium* strains (16,19,27) whose taxonomic positions have not yet been defined. It is the aim of this work to define the genetic relationships of these unclassified bacteria and to describe some of their characteristics and their symbiotic interactions with bean.

Rhizobium strains were isolated from legumes native to areas where *P. vulgaris* bean is widely cultivated (19). Rhizobial isolates from nodules from tropical plants such as *Dalea leporina*, *Clitoria ternantea*, *Leucaena leucocephala*, *L. esculenta*, and *Macroptilium gibbosifolium* were all capable of nodulating and fixing nitrogen in *P. vulgaris* bean. By the analysis of *nif* gene RFLP's and by their

legume host range they were considered different from *R. etli* as they had a broad host range and they had single *nif* operons (16,19).

Nucleotide sequence of 16S rRNA genes. In this work we determined the partial nucleotide sequence of the 16S rRNA genes of *R.* spp. strains CFN234 isolated from *Leucaena leucocephala*, CFN244 from *Macroptilium gibbosifolium*, CFN265 from *Leucaena esculenta* and Cli80 from *Clitoria ternantea* (Table 1), by direct sequence of polymerase chain reaction products. We also sequenced the same ribosomal gene fragment from *R.* spp. strain BR816 (44) which was isolated from *Leucaena leucocephala* nodules in Brazil. DNA sequences are available under GenBank accession numbers . A DNA region corresponding to nucleotides 20 to 338 of the *Escherichia coli* 16S rRNA was amplified from each strain using primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y2 (5'CCCACTG CTGCCTCCCGTAGGAGT-3') as described (49). DNA sequence was performed using a T7 sequencing kit from Pharmacia LKB. A multiple aligment was obtained using the PILEUP program of the Genetics Computer Group Sequence Analysis Package (7). A Jukes and Cantor Distance Matrix was constructed (12). The Neighbor Joining algorithm from the Neighbor program from Felsenstein's Phylip 3.5 (12) was used to determine the phylogenetic relationships (25). We rerooted the tree using the program RETREE from the aforementioned program package (12). Sequences of FL27 and OR191 were obtained from (9).

Accession number of analyzed sequences were as in (36). The tree (Fig. 1) is in general agreement with others previously reported (47,49). *R.* spp. form a cluster of bacteria with similar 16S ribosomal gene sequences, close to *R. etli* and to OR191. The latter was isolated in the USA from alfalfa ineffective nodules and nodulates effectively *P. vulgaris* (9). BR816 which has a different geographical origin is not clustered with these bacteria. BR816 is close to *R. meliloti*.

The phylogenetic analysis performed revealed that *R. etli* and *Rhizobium* spp. from tropical meso-american legumes probably shared a common ancestor. Other results also support their chromosomal relationship since *R.* spp. and *R. etli* all have a common isoelectric form of GSII (38). GSII is proposed to be used as a marker of species or groups of strains in the Rhizobiaceae (39).

Structural and functional comparison of *R.* spp. and *R. etli* sym plasmids. *R. etli* seems to be the specific symbiont of the common bean *P. vulgaris*. *R. etli* strains have a characteristic *nif* gene organization with multiple copies (30) and a narrow host range (16,19). In contrast *R. etli*-related strains have single *nif* gene operons and a broad host range, indicating perhaps that they harbor different or highly divergent symbiotic plasmids from *R. etli*. To verify this, the symbiotic plasmid from CFN249 transferred to the *Agrobacterium tumefaciens* plasmid-less strain GMI9023 (4) was purified and used as a probe for hybridization. A limited number of bands was obtained when *R. etli* CFN42 reference strain was

analyzed. Similarly, the *R. etli* sym plasmid was hybridized with total DNA-restriction fragments from *R.* spp. strains (Fig. 2a). Hybridization is observed to some extent but lower, in comparison to the large homology obtained with the *R. etli* strain tested (Fig. 2b).

Rhizobium and *Bradyrhizobium* species elicit the formation of nodules through signal molecules that are lipo-oligosaccharides of N-acetyl glucosamine (6,14). Different *Rhizobium* and *Bradyrhizobium* species produce signal molecules with different chemical substituents (23,29,31,33,37,42). We analyzed nod-metabolites from *R.* spp. CFN234, CFN249 and FL27; from *R. etli* CFN42 and *R. tropici* CFN299. Bacteria were grown in 1 ml minimal medium cultures (22,28) in presence or absence of flavonoid inducers and labeled with C^{14}-glucosamine (0.2 µCi/ml) or with S^{35}-sulfate (2 µCi/ml) for 12 hrs. Cultures were centrifuged and supernatants were chromatographed through C-18 Sep Pack cartridges. Fractions eluted with methanol were dried and used for thin layer chromatography HPTLC in direct-phase using chloroform, methanol and 5N ammonia (3:3:1 v/v). Autoradiographies are presented in Fig. 3. *R.* spp. produced oligosaccharides that were labelled both by C^{14}- glucosamine and by S^{35}- sulfate when induced by apigenin. Metabolites produced by *R.* spp. CFN234 are shown in Fig. 3. *R. etli* strain CFN42 produces non-sulfated compounds when induced by naringenin and genistein normally found in bean exudates (10). Differences in nod-metabolites are

in agreement to the differences observed between *R. etli* and *R.* spp. sym plasmid. Sulfated oligosaccharide signal molecules were first described for *R. meliloti* (14) and the sulfate modification is determinant for alfalfa host-specificity (31). Sulfate has a role in protecting from plant-chitinases (34). The broad-host rhizobia NGR234 and *R. tropici* also produce signal molecules bearing sulfate (20,28,29). Only the sulfated Nod-metabolites from *R. tropici* are capable to elicit true nodules in bean (20). Interestingly, production of sulfate - modified oligosaccharides is scattered in *Rhizobium* phylogeny.

We also evaluated *R.* spp. for melanin production which is a general characteristic of *R. etli* strains and is plasmid encoded (2). *R.* spp. strains CFN234, CFN244, Cli80, FL27 and CFN249 did not produce melanin or any other dark pigment in semisolid PY with tryptophan and Cu (2).

DNA-DNA homologies. *R.* spp. differed from *R. etli* strains not only in regard to the sym plasmid but also in regard to total number of plasmids. *R. etli* strains have on the average 3-4 plasmids while *R.* spp. normally have one plasmid or at most two (not shown). The differences in plasmid content may explain to some extent the differences in DNA-DNA hybridization obtained between *R. etli* and *R.* spp. It is estimated that plasmid content in *R. etli* strain CFN42 may represent around 25% of the genome. DNA-DNA hybridization was performed as described (22) using DNA from *R. etli* CFN2001 (35), a cured derivative of

plasmid a and psym from CFN42, as a probe. DNA homology is presented in Table 2. Within *R. etli* strains DNA-DNA hybridization is above 59% (35). In spite of the genetic resemblance revealed by the partial sequence of ribosomal genes, general DNA-homology is not considerable between *R. etli* and *R.* spp. (Table 2).

Nodulation and competition abilities of *R.* spp. Bean-nodulation capacities of *R.* spp. CFN244, CFN265, CFN249 and Cli80 were evaluated in vermiculite jars and in agar flasks as described (17,21) using Fahraeus N-free medium. Nodule number and nodule dry weight were on the average 20-50% of the nodule number normally obtained with *R. etli* type strain CFN42. Delay in nodulation was also observed (data not shown). Nitrogen fixation was determined by the acetylene reduction procedure (19). All nodules fixed nitrogen. In some cases specific nodule nitrogen fixing activity was similar in *R.* spp. induced-nodules and in those produced by *R. etli* CFN42. Although bean nodulation ability is widespread in these bacteria they are not as efficient for nodulation as the specialized *R. etli*. Competition for nodule formation was evaluated with different *R.* spp. and CFN42 in equal ratios as described (21). *R. etli* out-competed CFN244, CFN265 and CFN249, which could not form a single nodule under these conditions. This probably reflects what would happen in soil in normal bean crops as we were not able to isolate *R.* spp. from bean nodules but only *R. etli* strains (19). However we showed earlier that non-specific rhizobia may nodulate bean in soil if the former

exist in large numbers under specific conditions (19).

Ecogeographical relations of plants and rhizobia. *Leucaena* trees and *P. vulgaris* bean originated and diversified in Mesoamerica in similar habitats. Both crops have been introduced from Mexico and from other Latinoamerican countries to other regions *e.g.* bean to Europe and Africa and *Leucaena* to Hawaii (3), and Philippines. Some *R. loti* strains have been described to nodulate *Leucaena* spp. (11) Based on this, some strains from *Leucaena* have been assumed to be *R. loti* (26,43). Our results show that at least for Meso-american *Leucaena* rhizobia, this is not the case, *R. loti* is not a close relative of *R. etli*.

The ability to nodulate *P. vulgaris* is widespread among rhizobia. Nodulation of *P. vulgaris* by a wide range of strains from tropical legumes was reported by Lange in 1961 (13). *R. fredii* (32), *R. meliloti* (5) and other *R.* spp. (1) were reported to nodulate bean. Promiscuous nodulation has been reported for *Leucaena* (5) and its symbionts have also been reported to be heterogenous (24,41). *Leucaena* spp. belong to the Mimosoideae subfamily, and *P. vulgaris* belongs to the Papilonoideae subfamily of the Leguminosae. Interestingly their symbiotic bacteria are phylogenetically related.

In this work we defined a cluster of genetically related bacteria, that may be useful to understand evolutionary trends in rhizobia. Our results support the hypothesis (15) that symbiotic associations evolved between geographically limited

populations of legumes and soil bacteria having a single origin.

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FIG. 1. Phylogenetic tree obtained by the Neighbor-joining grouping derived from a Jukes-Cantor distance matrix of the aligned sequences of 16S ribosomal DNA-fragments of *Rhizobium* spp. and related bacteria. 1.3 cm is equal to 0.01 of genetic distance.

FIG. 2. Sym-plasmid homologies in *R.* spp. and *R. etli*. Autoradiogram of the Southern blot of EcoR1-digested genomic DNA of different *Rhizobium* strains. hybridized with a) *R.* sp. CFN249 sym plasmid. Lanes 1) CFN42; 2) CFN249 and with b) *R. etli* CFN42 sym-plasmid. Lanes 1) *R.* sp. CFN42; 2) *R.* sp. Cli80; 3) *R.* sp. CFN234; 4) *R.* sp. CFN265; 5) *R.* sp. CFN244. Sym plasmids were directly isolated from gels from *Agrobacterium tumefaciens* plasmid-less strain harboring CFN42 pSym or CFN249 pSym. Sym Plasmids were labeled with ^{32}P by nick translation as described (18) to be used as probes.

FIG. 3. Nod-metabolites from *R. etli* CFN42, (lanes 1-4), *R.* spp. CFN234 (lanes 5-8) and *R. tropici* CFN299 (lanes 9-12). Odd numbers show bacteria induced by flavonoids. Lanes 1,2,5,6,9 and 10 are factors derived from bacteria grown in S^{35}-sulfate. Lanes 3,4,7,8,11 and 13 are from rhizobia grown in C^{14}- glucosamine. Nod-metabolites are indicated with dots.

g.1

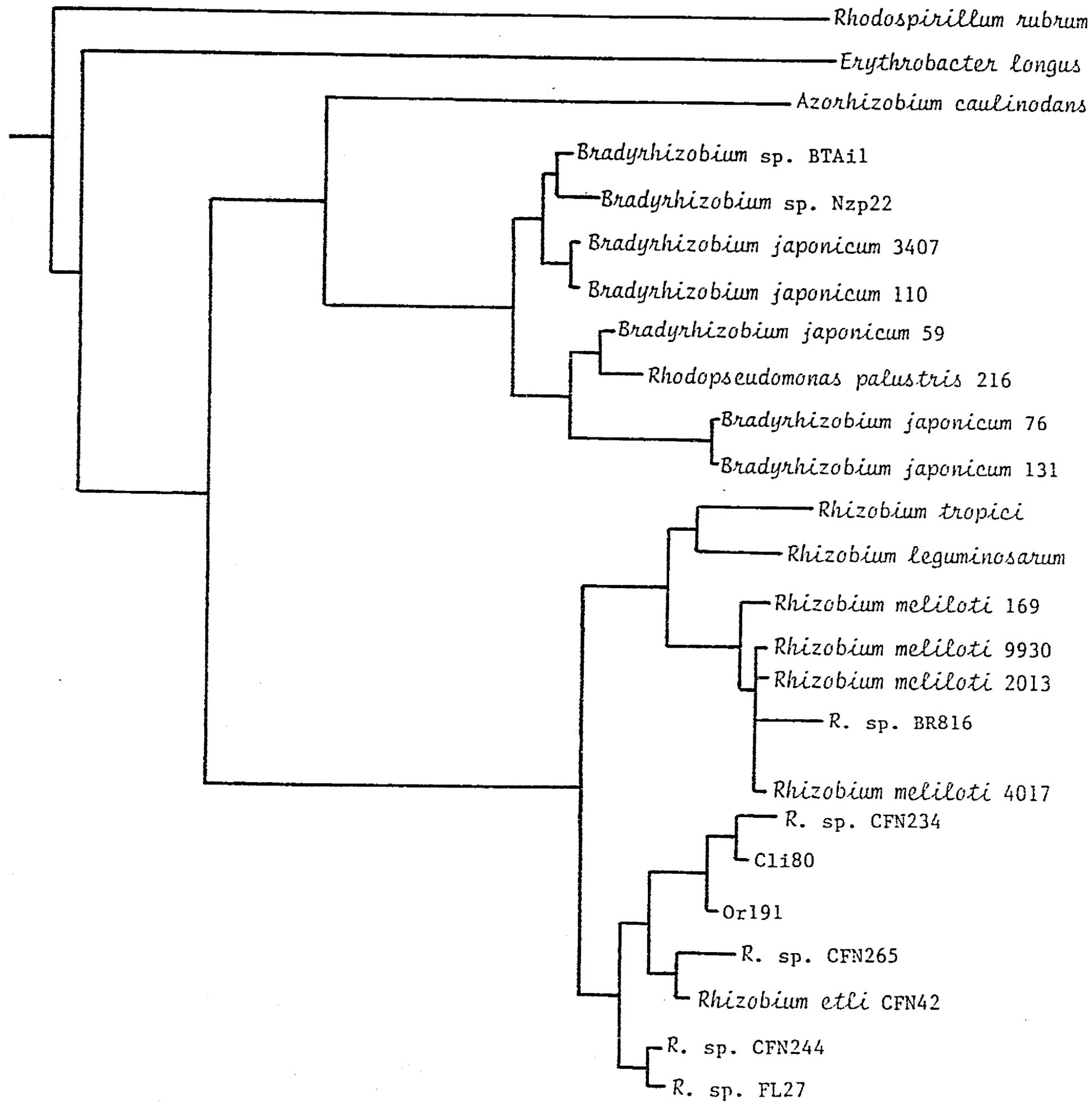
Rhodospirillum rubrum
Erythrobacter longus
Azorhizobium caulinodans
Bradyrhizobium sp. BTAi1
Bradyrhizobium sp. Nzp22
Bradyrhizobium japonicum 3407
Bradyrhizobium japonicum 110
Bradyrhizobium japonicum 59
Rhodopseudomonas palustris 216
Bradyrhizobium japonicum 76
Bradyrhizobium japonicum 131
Rhizobium tropici
Rhizobium leguminosarum
Rhizobium meliloti 169
Rhizobium meliloti 9930
Rhizobium meliloti 2013
R. sp. BR816
Rhizobium meliloti 4017
R. sp. CFN234
Cli80
Or191
R. sp. CFN265
Rhizobium etli CFN42
R. sp. CFN244
R. sp. FL27

Fig. 2

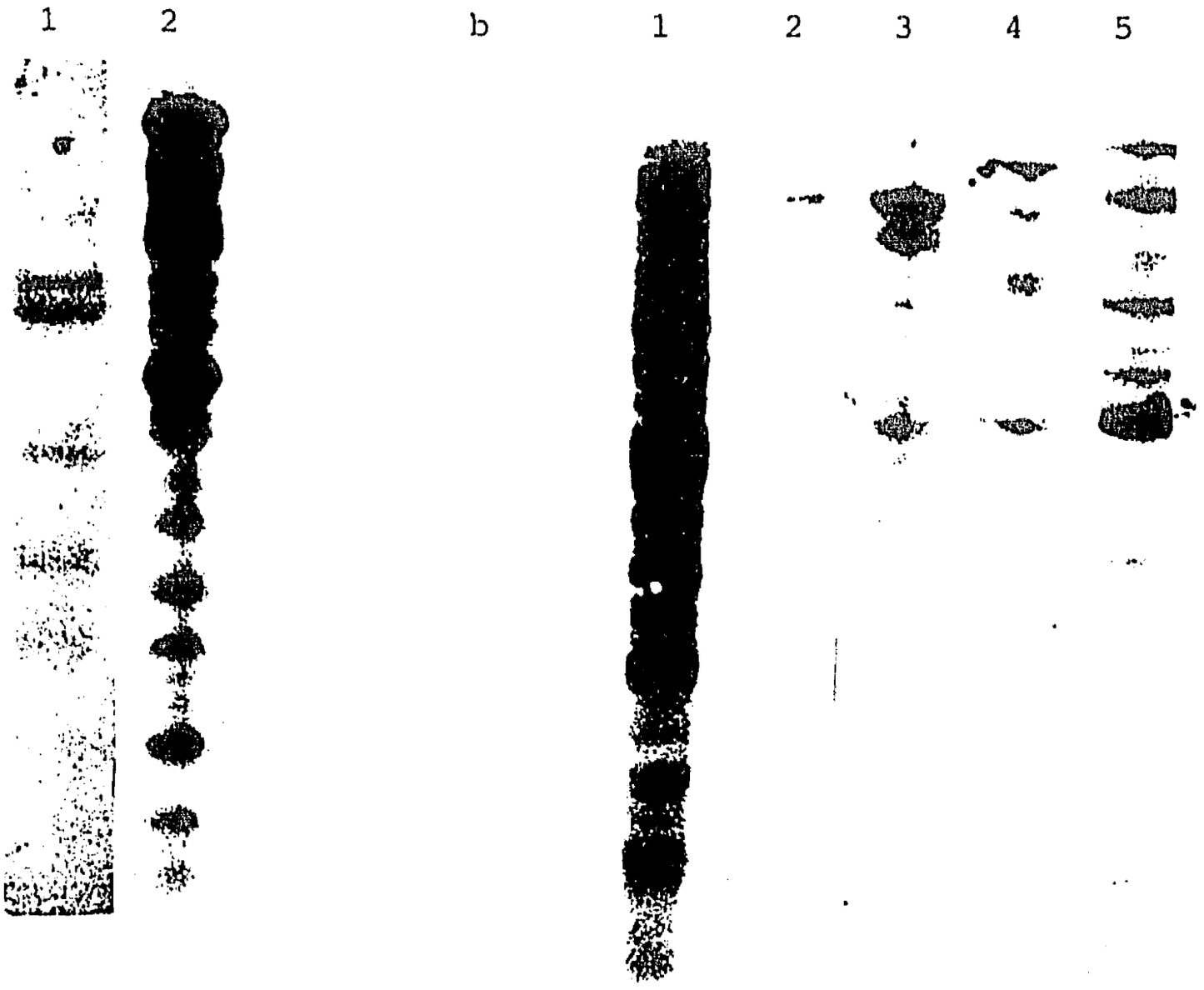

Fig. 3