TESIS PARA OBTENCIÓN DEL GRADO DE DOCTOR EN INVESTIGACIÓN BIOMÉDICA BÁSICA

TÍTULO: Ecología de *Acetobacter diazotrophicus* y biodiversidad de acetobacterias fijadoras de nitrógeno.

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Abstract

It has been shown that nitrogen-fixation could contribute up to 80% of the nitrogen requierements in sugarcane crops. *A. diazotrophicus* has been considered as a candidate for the N_2-fixation activity in that plant. This thesis focused on different items on the interaction of *A. diazotrophicus* and sugarcane. We determined the effect of the addition of nitrogen fertilizer on the plant colonization by *A. diazotrophicus*. The numbers of *A. diazotrophicus* inside stems and roots of inoculated plants that were fertilized with different nitrogen doses were determined by the most probable number. We also attempted to clarify the pattern of colonization of sugarcane by *A. diazotrophicus*. Tissues of plants inoculated with a ß-glucuronidase marked strain were analysed by microscopy and by scanning electron microscopy. Additionally, we attempted to contribute to the study of the nitrogen-fixation in sugarcane by using a reporter gene approach. We tried to determine if an *A. diazotrophicus* strain carrying a fusion of the dinitrogenase reductase promoter (*nifH*) with ß-glucuronidase showed activity in inoculated plants. We did not detect ß-glucuronidase activity in our experimental conditions. This thesis contributed to the knowledge of the genetic diversity of *A. diazotrophicus* and to the diversity of nitrogen-fixing acetobacters. We proposed two new species living in the coffee plant environment. We designed primers that could be used to identificate isolates belonging to that species. The use of these primers in PCR, specifically amplify a fragment of the 16S gene. In addition, the method for quantification of *A. diazotrophicus* cells associated to sugarcane plants was optimized by PCR, using the species-specific primer.

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RESUMEN

La asociación *Acetobacter diazotrophicus*-caña de azúcar surgió como un modelo importante de estudio ya que en caña de azúcar se había documentado contribución hasta del 80% de los requerimientos de nitrógeno de la planta por fijación biológica de nitrógeno (FBN). Se consideraba a *A. diazotrophicus* como el posible responsable de esta fijación y por eso en esta tesis se planteó abordar distintos aspectos de la asociación *A. diazotrophicus*-caña de azúcar. Se determinó el efecto de la adición de fertilizante nitrogenado en la colonización de la planta por *A. diazotrophicus*. La población de la cepa inoculada se cuantificó en el interior de plantas cultivadas con dos dosis diferentes de fertilización nitrogenada, utilizando el método del número más probable. Así mismo, tratando de contribuir a la determinación del patrón de colonización por *A. diazotrophicus*, se realizó microscopía óptica y electrónica de barrido de plantas con una cepa marcada con el gene reportero de la ß-glucuronidasa. En un acercamiento al estudio de la FBN por *A. diazotrophicus*, inoculamos plantas de caña con una cepa con expresión de ß-glucuronidasa dependiente del promotor del gene de la dinitrogenasa reductasa (*nifH*). Sin embargo, en nuestro sistema no detectamos expresión. Esta tesis contribuyó al estudio de la diversidad de *A. diazotrophicus* y de las acetobacterias fijadoras de nitrógeno. En este punto propusimos dos nuevas especies asociadas al ambiente de la planta de café. Entre los ensayos para la identificación de estos organismos sugerimos un grupo de oligonucleótidos que pueden amplificar específicamente un fragmento del gene 16S. Se optimizó el método de cuantificación de *A. diazotrophicus* utilizando el oligonucleótido específico en reacción de PCR.

INTRODUCCIÓN

Los seres vivos pueden utilizar diversos compuestos como fuente de nitrógeno. Sin embargo, el proceso mediante el cual se incorpora el nitrógeno atmosférico (fijación biológica de nitrógeno) se encuentra de manera exclusiva en organismos procariontes. Probablemente debido a esa situación, de manera natural se han establecido asociaciones entre bacterias fijadoras de nitrógeno con organismos que no pueden llevar a cabo esta actividad.

Como ejemplo de estas asociaciones se encuentran las de ciertas leguminosas con microorganismos fijadores de N_2 de la familia Rhizobiaceae y las asociaciones conocidas como actinorrizas, las que se establecen entre distintas familias vegetales y especies bacterianas de la familia Frankiaceae. Estas bacterias fijadoras de nitrógeno pueden colonizar a hospederos suceptibles y algunas también pueden habitar de manera libre.

En las asociaciones que se establecen entre distintas Rhizobia y plantas leguminosas se observa que durante el proceso de colonización las bacterias migran hacia tejidos específicos, en los que se establecen y sufren un proceso de diferenciación, dando lugar a células modificadas en su morfología y metabolismo (bacteroides). En la planta también se llevan a cabo modificaciones que conducen al establecimiento de la asociación simbiótica. La presencia de microorganismos específicos con la capacidad de infectar a la planta origina multiplicación celular localizada dando lugar a la formación de las estructuras recipientes de los bacteroides (nódulos).

El tejido de los nódulos se desarrolla después de la sucesión de una compleja serie de eventos de efector-respuesta entre ambos simbiontes. En esta interacción la liberación de ciertos compuestos químicos por uno de los organismos es detectada por su contraparte. Este último a su vez responde liberando otro metabolito o bien

produciendo los cambios morfológicos que dan lugar a la estructura nodular. El desarrollo del proceso de la nodulación ha sido abordado en estudios llevados a cabo en diversas asociaciones (Benson y col., 1993; Boivin y col., 1997; Guan y col., 1995; Hirsch, 1992; Schultze y col., 1994). En los nódulos se producen las interacciones planta-microorganismo que finalmente llevan a la obtención de compuestos de carbono por parte de la bacteria y de compuestos de nitrógeno por parte de la planta.

Las asociaciones *Rhizobium*-leguminosa y las actinorrizas representan sólo una proporción de la diversidad de relaciones ecológicas existentes entre fijadores de nitrógeno y otros organismos. La mayoría de las interacciones restantes han sido escasamente exploradas y aún cuando en algunos casos se ha observado un efecto promotor del crecimiento de la planta asociada, no se tiene certeza de que ésto se deba al aporte de compuestos nitrogenados por parte de la bacteria. De las asociaciones menos estudiadas están las que se establecen entre las plantas y las bacterias endófitas, en particular las asociaciones con endófitos no patógenos. Estas bacterias se localizan en el interior de ciertos tejidos vegetales. A diferencia de las especies de las familias Rhizobiaceae y Frankiaceae, estos microorganismos no forman estructuras específicas y su localización es más dispersa. *Acetobacter diazotrophicus* es una bacteria endófita fijadora de nitrógeno que originalmente se aisló de la planta de caña de azúcar (Cavalcante y Döbereiner, 1988). En medio de cultivo *A. diazotrophicus* tiene capacidad de fijar nitrógeno aún en condiciones que resultan inhibitorias para otros diazótrofos, tales como pH menor a 3.0, alta osmolaridad en sacarosa o glucosa (Cavalcante y Döbereiner, 1988) y concentraciones relativamente altas de amonio y de oxígeno (Stephan y col., 1991). Este microorganismo se ha aislado del interior de la planta de la caña de azúcar (Cavalcante y Döbereiner, 1988; Fuentes-Ramírez y col. 1993; Li y MacRae, 1991; Reis y col., 1994), y en una menor frecuencia de su rizósfera (Li y MacRae, 1991). *A. diazotrophicus* también se ha aislado del interior de esporas de hongos micorrízicos, provenientes de suelo donde

crece la caña de azúcar (Velázco y col., 1990), así como de un insecto (Orden Homoptera) que se alimenta de fluídos de esta planta (Ashbolt e Inkerman, 1990; Caballero-Mellado y col., 1995 [Anexo II]). El aislamiento de *A. diazotrophicus* en ambientes distintos a la caña de azúcar ha permitido ampliar el conocimiento de la distribución de esta bacteria. Se conoce que *A. diazotrophicus* se desarrolla en asociación con distintas gramíneas (Döbereiner y col., 1993; Loganathan y col., 1999); también se le encuentra en la planta de café y en la rizósfera de este cultivo (Jiménez-Salgado y col., 1997, [Anexo III]); y en asociación con la planta de piña (Tapia-Hernández y col., 2000).

A. diazotrophicus ha sido considerado como una bacteria endófita debido a que en suelo no rizosférico su abundancia es mínima (Li y MacRae, 1992); su supervivencia se reduce notablemente en sustrato pobre en fuentes de carbono (Boddey y col., 1995; Fuentes-Ramírez, L. E., Jiménez-Salgado, T., Abarca-Ocampo, I. y Caballero-Mellado, J., resultados sin publicar); y a que no se ha detectado actividad patogénica en la asociación con el vegetal.

En distintos estudios de localización de *A. diazotrophicus* en la caña de azúcar (Dong y col., 1994; Dong y col., 1997; James y col., 1994) se reportan resultados diferentes. En plántulas micropropagadas de caña de azúcar inoculadas, James y col. (1994) detectaron colonización por *A. diazotrophicus* en epidermis de raíz y en xilema de tallo, en su parte basal, utilizando microscopía óptica (MO), microscopía electrónica de barrido (MEB), microscopía electrónica de transmisión (MET) e inmunodetección microscópica en secciones de planta. Los autores sugieren la posibilidad de la translocación de *A. diazotrophicus* a lo largo de la planta a través de los vasos del xilema, así como la potencialidad de este tejido para mantener en su interior poblaciones bacterianas metabólicamente activas. Por su parte, Dong y col. (1994 y 1997) reportaron la detección de *A. diazotrophicus* únicamente en los espacios intercelulares de tallos de plantas de caña de azúcar inoculadas y sin inocular. Los

autores aislaron *A. diazotrophicus* a partir de muestras de fluido apoplástico, resultado que relacionaron con la detección microscópica de células bacterianas en los espacios intercelulares. En los trabajos ya citados de Dong y col. se sugiere que *A. diazotrophicus* no coloniza el tejido vascular de la caña de azúcar, conclusión que es diferente a la referida por James y col. (1994) y por trabajo nuestro (Fuentes-Ramírez y col., 1999, [Anexo I]).

Se han reportado efectos benéficos en el desarrollo de distintas plantas por la asociación de éstas con *A. diazotrophicus* (Isopi y col., 1995; Paula y col., 1992; Sevilla y col., 1998). En experimentos con camote dulce (*Ipomoea batatas*) inoculado con *A. diazotrophicus* y hongos micorrízicos se detectó aumento tanto en el crecimiento de la planta como en el contenido de nitrógeno y fósforo, en comparación con plantas inoculadas con la bacteria o con el hongo, por separado (Paula y col., 1992). La promoción de crecimiento y de estado nutricional de la planta probablemente fue resultado directo de la asociación con la micorriza ya que no se detectó contribución de nitrógeno proveniente de fijación biológica. Aparentemente, *A. diazotrophicus* facilitó la micorrización de la planta, incrementado la translocación de nutrientes por el hongo asociado (Paula y col., 1992). En otro estudio llevado a cabo con sorgo, se observaron incrementos en el contenido de nitrógeno en el tallo de plantas inoculadas con *A. diazotrophicus*, así como de plantas inoculadas con esta misma bacteria y con distintas especies de hongos micorrízicos (Isopi y col., 1995).

Las cepas de *A. diazotrophicus* aisladas del interior de la caña de azúcar muestran una diversidad genética muy baja en comparación a la diversidad de poblaciones de otras especies bacterianas que se desarrollan en vida libre (Martínez-Romero y Caballero-Mellado, 1996). En opinión de los autores, este bajo grado de diversidad podría deberse a la estabilidad de condiciones ecológicas que se presenta en el interior de la planta, a semejanza de lo que se sugiere respecto a la diversidad de bacterias fitopatógenas (Denny y col., 1988; Gabriel y col., 1988; Hartung y Civerolo,

1989; Leite y col., 1994; Scholz y col., 1994).

La abundancia de las poblaciones de *A. diazotrophicus* parece estar ligada a la concentración de nitrógeno combinado del suelo en el que crece la planta, así se ha observado que la cantidad de aislamientos de *A. diazotrophicus* obtenidos a partir de plantas de caña crecidas con altas dosis de fertilizantes nitrogenados es menor, en comparación a la cantidad de aislamientos que se obtienen a partir de plantas crecidas con menor cantidad de nitrógeno (Fuentes-Ramírez y col., 1993; Muthukumarasamy, 1998). Aunado a lo anterior, se ha observado que las poblaciones aisladas de plantas de caña de azúcar cultivadas en regiones donde se ha aplicado más fertilizante nitrogenado son las que exhiben menor diversidad genética (Caballero-Mellado y col., 1995, [Anexo iii]).

La presencia de nitrógeno combinado (NO_3^- 10 mM) no inhibe el crecimiento de distintos genotipos de *A. diazotrophicus* en medios de cultivo. La existencia de sólo ciertos genotipos bacterianos en plantas de caña de azúcar altamente fertilizadas con nitrógeno podría estar mediada por una selección de parte del vegetal. Las condiciones de fertilización nitrogenada podrían influir en el estado fisiológico particular de la planta (Caballero-Mellado y Martínez-Romero, 1994; Caballero-Mellado y col., 1995; Martínez-Romero y Caballero-Mellado, 1996). En este sentido, se conoce que niveles elevados de nitrógeno en la rizósfera retrasan el crecimiento vegetal, probablemente a causa del aumento en el consumo de la energía utilizada para la absorción e incorporación de este nutriente (Barnes y Hole, 1978). Durante la incorporación en el corto plazo de compuestos inorgánicos nitrogenados, en especial NO_3^-, tanto en hojas de plantas de fotosíntesis tipo C_3 como de tipo C_4, se utiliza poder reductor que puede provenir tanto de elementos reductores tales como ferredoxinas, como de moléculas reductoras sintetizadas en la fijación de CO_2 (Champigny, 1995). Este desvío de electrones trae como consecuencia la disminución en la síntesis de sacarosa debido a una reducción en la concentración de precursores. Por otra parte, las actividades enzimáticas que

intervienen en la síntesis de sacarosa, probablemente también son influidas por el estado nitrogenado de la planta (Champigny, 1995).

No obstante la clara relación del nitrógeno con el metabolismo del carbono, no se tiene un esquema que pueda aplicarse a cualquier circunstancia. Como ejemplo, Peláez-Abellán y col. (1994) determinaron en caña de azúcar el efecto de la concentración de nitrógeno en la síntesis de sacarosa y en la actividad de fosfoenol-piruvato carboxilasa (PEPcasa). Para ello utilizaron explantes de hoja de caña de azúcar que habían sido incubados por periodos cortos en presencia de dos concentraciones distintas de nitratos. En una de las variedades utilizadas observaron disminución en la síntesis de sacarosa en los cortes foliares expuestos a nitratos, mientras que en los cortes de la otra variedad hubo una aparente estimulación en la síntesis de sacarosa bajo las mismas condiciones. En cuanto a la actividad de PEPcasa, en la variedad que mostró disminución en la síntesis de sacarosa no se detectaron cambios debidos a la presencia de nitratos, mientras que en la variedad que había mostrado incremento en la síntesis de sacarosa si se detectó un incremento en PEPcasa al aumentar la concentración de nitratos. Otras respuestas de la planta a la presencia de compuestos nitrogenados se dan en la fijación de CO_2. En distintas plantas con fotosíntesis C_4 se ha observado que el incremento en la concentración de nitrógeno combinado aumenta el punto de compensación de CO_2 sensible a O_2 y disminuye la tasa fotosintética por inhibición de O_2 (Cresswell y col., 1979). Gomes y Crocomo (1991) detectaron disminución en la concentración de azúcares reductores y de sacarosa en tallo de plantas de caña de azúcar cultivadas con un alto régimen nitrogenado.

Las variedades actuales de caña de azúcar proceden de cruzas interespecíficas de especies del género *Saccharum* (*S. officinarum* L., *S. spontaneum* L. y *S. robustum* Jeswiet), las que son originarias de Asia y de las islas del Pacífico Sur. El cultivo de la caña se ha ido extendiendo a diversas regiones tropicales desde hace unos cuatro

siglos. Las variedades comerciales actuales son el fruto de la hibridación y la selección fenotípica que se ha llevado a cabo en distintos puntos geográficos, por lo que las condiciones empleadas en cada una de estas regiones han influido en las características de cada variedad. En Brasil el cultivo de la caña es atípico con respecto a la mayoría de países, en relación a la cantidad de fertilizante nitrogenado que se utiliza. Así, en Brasil la selección de variedades se ha llevado a cabo con bajos niveles de nitrógeno en campo, a diferencia de la selección que se ha realizado en la mayoría de los países productores. Esto ha llevado de manera colateral a que muchas de sus variedades muestren una respuesta pobre a la adición de nitrógeno (Boddey, 1995; Ruschel, 1981). Si se toma en cuenta lo mencionado anteriormente, aunado al hecho de que la caña de azúcar puede extraer de 100 a 200 Kg de N por hectárea y de que en Brasil este cultivo normalmente se fertiliza con cantidades de este nutriente que van de los 60 a los 120 Kg por hectárea, es probable que el nitrógeno restante provenga de la fijación de nitrógeno (Boddey y col., 1991).

Las primeras evidencias directas de fijación biológica de nitrógeno (FBN) en la caña de azúcar se obtuvieron en plantas desarrolladas en atmósfera enriquecida con $^{15}N_2$ (Ruschel y col., 1975). Matsui y col. (1981) reportaron FBN en un experimento en el que introdujeron $^{15}N_2$ en la región radical de caña de azúcar. De manera similar, Ruschel y col. (1981) detectaron FBN en esta misma planta aún en presencia de nitratos, después de exponer la raíz de plantas intactas a $^{15}N_2$. Posteriormente se ha determinado la contribución de la FBN a la caña de azúcar durante el ciclo de vida de la planta. Los resultados obtenidos con distintas variedades de caña de azúcar utilizando dilución isotópica con ^{15}N, análisis de balance de nitrógeno y comparación de abundancia natural de ^{15}N han sugerido aportes de hasta 70% del nitrógeno total por FBN, aunque también se han detectado variedades que mostraron menor o nula actividad fijadora de nitrógeno (Lima y col., 1981; Vose y col., 1981; Yoneyama y col., 1997).

Las condiciones ideales de pH y de concentración de azúcares para el crecimiento y para la expresión *in vitro* de la actividad fijadora de nitrógeno por parte de *A. diazotrophicus*, son semejantes a las condiciones que se encuentran en el interior de la caña de azúcar (Cavalcante y Döbereiner, 1988; Hartmann y col., 1991; Stephan y col. 1991). A partir de esos datos, se ha sugerido que la caña podría ser directamente beneficiada por aporte de nitrógeno proveniente de la actividad de *A. diazotrophicus*. En experimentos de inoculación de plántulas de caña de azúcar se reportaron efectos positivos en el crecimiento debidos a la inoculación con *A. diazotrophicus* (Sevilla y col., 1998). En este trabajo se observó promoción del crecimiento vegetal al inocular una cepa silvestre en plantas cultivadas en condiciones de limitación de nitrógeno combinado, pero no cuando las plantas se inocularon con una mutante *nifD*. Sin embargo, también se detectaron efectos positivos por inoculación con las dos cepas cuando las plantas se desarrollaron con abundancia de fertilizante nitrogenado, lo que sugiere que al menos en parte el aumento en el crecimiento detectado pueda deberse a factores diferentes a la FBN, tales como la promoción del crecimiento radical y consecuentemente el incremento en la absorción de minerales, vía la producción bacteriana de hormonas vegetales.

En un experimento en campo, Sevilla y col., (1999) obtuvieron resultados semejantes a los resultados anteriores de invernadero, además de que también detectaron incorporación de ^{15}N a partir de $^{15}N_2$ en plantas inoculadas con la cepa silvestre. Estos resultados sugieren fuertemente que la fijación por *A. diazotrophicus* podría estarse realizando en el interior de la planta. Sin embargo, la evaluación de la contribución de nitrógeno por *A. diazotrophicus* podrá determinarse sólo por el uso de metodologías que permitan cuantificar la fijación biológica durante la totalidad del ciclo de vida de la caña.

El conocimiento de la composición taxonómica de cualquier grupo de organismos es punto de partida para la investigación biológica tanto en áreas

fundamentales como aplicadas. Hasta recientemente, en la microbiología se carecía de metodologías adecuadas para el desarrollo de una taxonomía que reflejara la filogenia de las bacterias. Esta deficiencia metodológica fue la causa de que el estudio de la biodiversidad de los organismos procariontes estuviera severamente limitado por un largo periodo. El desarrollo reciente de nuevas herramientas procedentes del estudio molecular de los procesos que se llevan a cabo en los seres vivos ha contribuido, entre otros muchos campos, al avance de la taxonomía bacteriana. El conocimiento de la biodiversidad bacteriana actualmente está en continua revisión por la descripción de nuevas especies provenientes de ambientes diversos, entre los que se incluyen los ambientes del interior y del exterior de las plantas. La composición taxonómica de las bacterias asociadas a una gran cantidad de plantas está siendo apenas explorada. Dentro de la subclase α-Proteobacteria se encuentran varios géneros filogenéticamente cercanos que forman a la familia Acetobacteracea y que se incluyen dentro de las bacterias acéticas (Swings, 1992). La composición de esta familia ha sido recientemente modificada por la división del género *Acetobacter* (Validation list 64, 1998; Yamada y col., 1997) y por la descripción de una especie ubicada en un género nuevo (Yamada y col., 2000). A propuesta de Yamada y col. (1997), las especies *Acetobacter diazotrophicus*, *A. europaeus*, *A. hansenii*, *A. liquefaciens* y *A. xylinus* han sido recientemente ubicadas dentro de la misma familia Acetobacteraceae en el nuevo género *Gluconacetobacter* (Validation list 64, 1998). La propuesta de creación del género *Gluconacetobacter* se basó en diferencias en secuencia parcial del gene ribosomal 16S y en el tipo de ubiquinona que producen las especies anteriormente ubicadas en conjunto en el género *Acetobacter*. Consideramos que la creación de un nuevo género debería estar apoyada por un conjunto más amplio de rasgos fenotípicos y genotípicos, de manera que se refleje más fidedignamente la totalidad del genoma de los organismos. Debido a los motivos mencionados anteriormente y a que la especie fijadora de nitrógeno *Gluconacetobacter diazotrophicus* tradicionalmente ha sido

conocida como *Acetobacter diazotrophicus*, en esta tesis toda referencia a esa bacteria se hará con la denominación original.

La identificación de células de *A. diazotrophicus* asociadas con plantas se ha realizado por determinación de características fenotípicas de cepas aisladas utilizando medios de enriquecimiento y diferenciales (Dong y col., 1994; Fuentes-Ramírez y col., 1993); también por uso la técnica inmunológica de ELISA (Li y MacRae, 1992); y por hibridación y PCR utilizando oligonucleótidos específicos (Kirchhof y col., 1997b; Sievers y col., 1998). El método de identificación por aislamiento proporciona resultados muy confiables. Sin embargo, su principal inconveniente es el tiempo empleado ya que éste puede abarcar desde dos hasta cuatro semanas. La aplicación de ELISA y el uso de oligonucleótidos específicos permiten una reducción considerable en el tiempo empleado en la identificación taxonómica. Aún así, la especificidad de esas metodologías podría estar limitada en grado diverso, ya que por una parte tal especificidad depende de la muestra de cepas o biovares o especies a partir de las cuales se obtienen los anticuerpos o se diseñan los oligonucleótidos, y por otra parte es sabido que la diversidad en muy distintos grupos taxonómicos es conocida sólo parcialmente.

En esta tesis se abordaron varios temas sobre la ecología de *A. diazotrophicus*, éstos incluyen un estudio de la colonización en caña de azúcar, ecología de poblaciones y taxonomía de esta especie, y el desarrollo de herramientas y metodología para detectar y cuantificar de manera especifica células de *A. diazotrophicus* y organismos relacionados filogenéticamente.

OBJETIVOS GENERALES

Contribuir al conocimiento de la ecología de la colonización de la caña de azúcar por *A. diazotrophicus*.

Contribuir al conocimiento de la biodiversidad de las bacterias acéticas fijadoras de nitrógeno, asociadas a especies vegetales.

JUSTIFICACION Y METAS

Al inicio de este proyecto de tesis se contaba con escasos datos sobre distintos aspectos de la ecología de la relación entre *A. diazotrophicus* y la caña de azúcar, tales como los encaminados a establecer el patrón de colonización de *A. diazotrophicus* en la caña de azúcar y los efectos que sobre este patrón pudiera tener la fertilización nitrogenada de la planta. Las metas generales y específicas son:

Meta I.1 Determinar el efecto de la fertilización nitrogenada de la caña de azúcar en la colonización por *A. diazotrophicus*.

Específicamente, determinar la densidad de población de células de *A. diazotrophicus* en plantas de caña de azúcar inoculadas a través de esquejes propagativos y cultivadas con distintas dosis de fertilización nitrogenada.

Meta I.2 Localizar los sitios de colonización de *Acetobacter diazotrophicus* en caña de azúcar.

Construir una cepa de *A. diazotrophicus* Gus^+ constitutiva.

En plantas de caña de azúcar inoculadas a través de esquejes propagativos determinar por ensayo histoquímico e identificar macroscópicamente y por microscopía óptica los tejidos con expresión de actividad β-glucuronidasa.

Entre las especies diazótrofas que colonizan endofíticamente a la caña de azúcar, se ha sugerido que *A. diazotrophicus* pudiera fijar nitrógeno dentro de la planta debido sus características peculiares. Sin embargo, no había sido plenamente establecido si en asociación con la caña de azúcar *A. diazotrophicus* es un fijador de nitrógeno activo.

Al inicio de este proyecto de tesis se planteó la meta siguiente:

Meta II. Determinar si en asociación con la caña de azúcar se transcribe el gene *nifH* de *A. diazotrophicus*.

Específicamente, determinar la secuencia nucleotídica "upstream" del gene *nifH* de *A. diazotrophicus* y por análisis de ésta, detectar la región mínima necesaria a clonar que contenga a las siguientes regiones reguladoras putativas: promotor y "Upstream Activator Sequence" (UAS).

En un vector que se mantenga establemente en *A. diazotrophicus*, construir una fusión transcripcional *nifH::gusA* con la región reguladora de la dinitrogenasa reductasa del mismo microorganismo.

Determinar *in vitro* la actividad de la fusión *nifH::gusA* en cultivos de *A. diazotrophicus* bajo distintas concentraciones de nitrógeno combinado.

En plantas de caña de azúcar inoculadas a través de esquejes propagativos determinar por ensayo histoquímico si se presenta actividad β-glucuronidasa.

En muy distintos habitats se desarrollan comunidades bacterianas de las que sólo recientemente comenzamos a conocer su diversidad. En asociación al ambiente de la planta de café se han aislado poblaciones de *A. diazotrophicus* y de otras cepas fijadoras de nitrógeno relacionadas con esta especie. Nos propusimos definir la posición taxonómica de estas cepas con los siguientes objetivos específicos:

Meta III. Analizar la diversidad taxonómica de cepas de acetobacterias fijadoras de nitrógeno que se desarrollan en el ambiente rizosférico y endófito de la planta de café y, de ser posible, diseñar oligonucleótidos útiles para la identificación de cepas fijadoras de nitrógeno pertenecientes a las bacterias acéticas.

Como meta específica, determinar la secuencia del gene ribosomal de la subunidad pequeña de cepas de acetobacterias fijadoras de nitrógeno que se desarrollan en el ambiente de la planta de café.

Por comparación con las secuencias de los genes ribosomales de la subunidad pequeña de las especies reconocidas de la familia Acetobacteraceae determinar la relación filogenética de las cepas de interés.

A partir de regiones que muestren una alta variabilidad entre la familia Acetobacteraceae, diseñar oligonucleótidos útiles para amplificar parcialmente el gene ribosomal de la subunidad pequeña de cepas fijadoras de nitrógeno de la familia Acetobacteraceae de manera específica.

La cuantificación de la densidad poblacional de *A. diazotrophicus* constituye una faceta de estos estudios de la interacción entre la bacteria y la caña de azúcar. Estas determinaciones se llevan a cabo por cuenta de crecimiento a partir de la siembra de las diluciones de extractos de plantas en medios de enriquecimiento y verificación de identidad de la bacteria. El tiempo total para la cuenta de células de *A. diazotrophicus* en planta es de dos a tres semanas. Debido a ello, el disponer de procedimientos confiables que reduzcan la duración de la cuantificación bacteriana de *A. diazotrophicus*, constituiría un apoyo experimental valioso. Con la finalidad de contribuir a la optimización de la metodología utilizada en la determinación de cuenta poblacional de *A. diazotrophicus* se planteó:

Meta IV. Diseñar un método reproducible y que sea eficiente en cuanto al tiempo de realización, para la identificación y la cuantificación de células de *A. diazotrophicus* asociadas a plantas de caña de azúcar.

Determinar la posibilidad de cuantificar células de *A. diazotrophicus* utilizando el método del número más probable y PCR, de manera combinada, utilizando oligonucleótidos específicos diseñados a partir de la secuencia del gene ribosomal de la subunidad pequeña.

Determinar la confiabilidad del método por comparación con la cuantificación por número más probable en crecimiento en medio de enriquecimiento.

Determinar cuantitativamente la presencia de células de *A. diazotrophicus* en plantas de caña de azúcar inoculadas.

RESULTADOS

I. ECOLOGÍA DE LA COLONIZACIÓN

Los resultados sobre los efectos de la fertilización nitrogenada en las poblaciones de *A. diazotrophicus* en la caña y su localización (Meta I) se muestran en el artículo: "Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization" (Anexo I).

II. PROMOTOR DE *nifH* Y EXPRESIÓN DEL GENE.

Los resultados no publicados sobre el estudio de la expresión del gene *nifH* y su región regulatoria (Meta II) se presentan a continuación:

El aislamiento, secuenciación y análisis de la zona regulatoria del gene *nifH* se requirió para la construcción de un plásmido con un gene reportero de la expresión de nitrogenasa. Este plásmido (pRGH562) se introdujó en *A. diazotrophicus* UAP 5541 que se inoculó en plantas de caña de azúcar. Colateralmente la construcción UAP 5541/pRGH562 sirvió para estudiar la regulación de la expresión por amonio. Previamente, la fijación de nitrógeno de *A. diazotrophicus* se había reportado como altamente tolerante al nitrógeno fijado (Cavalcante y Döbereiner, 1988; Stephan y col., 1991).

Los cósmidos se obtuvieron a partir de ADN digerido parcialmente con la enzima *Sal*I y ligado al vector pSUP205 (Simon y col. 1983). Por hibridización con una sonda intragénica de *nifH* (fragmento *Sal*I-*Sal*I de 0.3 kb), procedente de *R. etli* (Morett y col. 1988) se identificaron cuatro cósmidos que compartían las mismas bandas de hibridación que el ADN genómico de la cepa *A. diazotrophicus* UAP 5560. Las bandas

de hibridación obtenidas al cortar ADN genómico de la cepa UAP 5560 con las enzimas de restricción *Bgl*II, *Pst*I, *Bam*HI, *Hind*III y *Kpn*I correspondían a los tamaños 12, 8, 7, 4.3 y 6 kb, respectivamente.

A partir del cósmido pHF38, que mostró un patrón de hibridación (*Eco*RI) de *nifHDK* idéntico al de la cepa UAP 5560, se aisló un fragmento *Bam*HI de 7 kb. El plásmido pNHAD7 se construyó por la inserción del fragmento *Bam*HI de 7 kb en pUC19. Se utilizaron subclonas del pNHAD7 para determinar la secuencia del promotor de *nifH* y de la región 5' del mismo gene (492 bases de la región regulatoria y 291 de la región codificante). La secuencia obtenida se depositó en el GenBank con el número de acceso U78044. A 192 bases arriba del gene *nifH* de *A. diazotrophicus* UAP 5560 se encontró una secuencia invertida repetida (IR) que abarca 31 nt y que teóricamente favorece la formación tallos de 14 nt (ΔG de -28.8 kcal/mol). Arriba del gene *nifH* se encontró un marco abierto de lectura de 55 codones, al que llamamos orf1 (Fig. 1). La comparación del orf1 con bancos de secuencias no mostró semejanza significativa con ningún gene reportado (no se muestra). Por comparación de secuencia se buscaron las regiones reguladoras que compartieran la mayoría de los genes de la dinitrogenasa reductasa de otros organismos. La organización que muestra la zona reguladora con la disposición de las secuencias detectadas coincide con la equivalente en la mayoría de fijadores de nitrógeno. A las distancias indicadas a partir del primer ATG se localizaron las siguientes secuencias putativas: sitio de unión a ribosoma a -8 nucleótidos, una secuencia GTGGCATGGCGTTTGCG que corresponde al consenso de los promotores -12 -24 (Morett y Buck 1989), un sitio de unión a IHF (integration host factor) de la base -81 a la -128, y un fragmento con el consenso TGT-N10-ACA (UAS, Upstream Activator Sequence), típico de los sitios unión a NifA (ver revisión Merrick, 1992). El aparente sitio de unión a IHF se localizó utilizando el programa Seqscan, disponible en la red en la dirección http://www.bmb.psu.edu/seqscan/default.htm, de B. T. Nixon.

A partir del pNHAD7 se amplificó un fragmento de 632 pb, que contenía la

región reguladora de *nifH* (Fig. 1). La secuencia del oligonucleótido que se une arriba de la región regulatoria fue 5'-CTCTCTAGAGGATCCCGGTCCGTATTATGCCGTCC-3', oligonucleótido "upnifH", y la del oligonucleótido que se acopla en la región estructural del gene *nifH* fue 5'-CTCCCCGGGGGACGCACTTGATGCCCTTGTA-3', oligonucleótido "nifH". El fragmento amplificado se digirió con las enzimas *Sma*I y *Bam*HI y se ligó en el vector pRG960SD (Van den Eede y col., 1992) produciendo una fusión del promotor de *nifH* con el gene reportero *gusA1* (plásmido pRGH562).

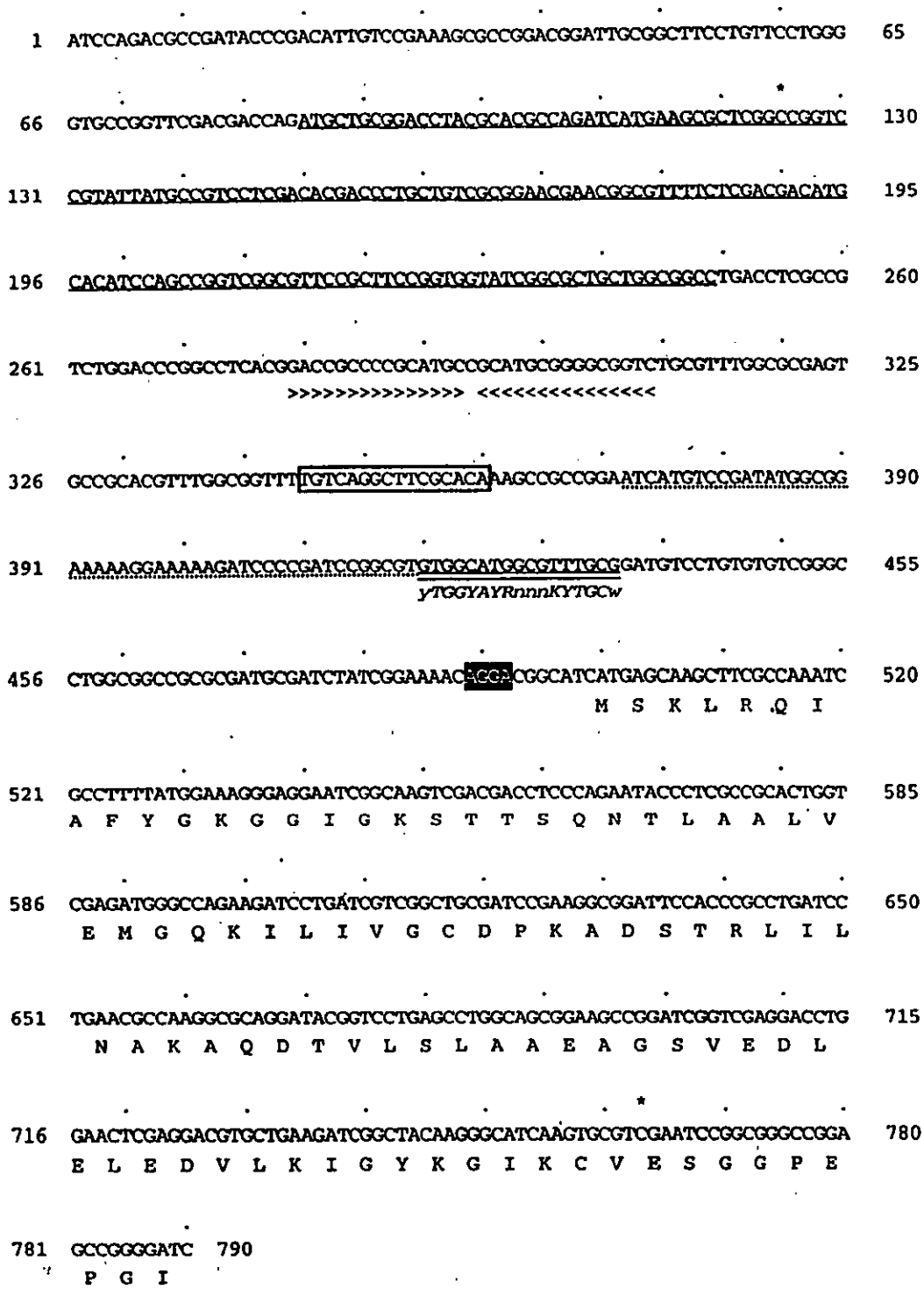


Figura 1. Secuencia nucleotídica de la zona regulatoria del gene *nifH* y del extremo 5' del mismo. Se muestran las siguientes regiones putativas: orf1, en subrayado; UAS, marcadas con una caja; sitio de unión a IHF, subrayado punteado; promotor dependiente de σ^{54}, en doble subrayado, el consenso se muestra en itálicas; y sitio de unión a ribosoma, en sombreado. Una secuencia invertida repetida (IR) se muestra con flechas. El fragmento amplificado por PCR y posteriormente clonado en el vector con el gene de *gusA* (pRG960SD) esta limitado por asteriscos.

Dos señales negativas para regulación transcripcional de *nifH* en diferentes bacterias diazotróficas no simbióticas son la disponibilidad de nitrógeno combinado y la presencia de oxígeno (ver revisiones de Elmerich, 1991; Merrick, 1992; y Merrick, 1993). Por ello probamos la influencia del amonio y del oxígeno en la expresión del promotor de *nifH* en medio de cultivo. La expresión del promotor de *nifH* de la cepa UAP 5560 se cuantificó en el fondo genético de la cepa UAP 5541 de *A. diazotrophicus*, utilizando una fusión transcripcional con el reportero *gusA* [plásmido pRGH562 (Tabla 1)]. Previo a este experimento se había determinado la ausencia de actividad β-glucuronidasa (GUS) en *A. diazotrophicus* por lo que la actividad GUS en la cepa trabajada procede exclusivamente de la construcción que se introdujo. La actividad del promotor de *nifH* esta regulada por la concentración de amonio en el medio. La expresión de *nifH* fue 80 veces más alta en condiciones de limitación de nitrógeno que en exceso de éste. En presencia de amonio se detectó sólo una ligera expresión residual del promotor, por lo que se puede eliminar la posibilidad de transcripción inducida por secuencias del vector.

Tabla 1. Regulación del promotor de *nifH* de *A. diazotrophicus* por amonio y oxígeno[a].

Plásmido[c]	Actividad de β-glucuronidasa[b]			
	sin NH_4^+		con NH_4^+	
	O_2 atm	O_2 1%	O_2 atm	O_2 1%
pRG960SD	3.0	7.0	3.2	5.0
pRGH562	4.0	913.0	2.8	84.0
pRGS561	2.7	39.5	3.4	66.8

[a]Las células bacterianas se cultivaron durante 48 h a 30°C en medio líquido SUCMES, con $(NH^+_4)_2SO_4$ 12 mM o en medio libre de nitrógeno, pH 6.5. Los recipientes fueron cubiertos con tapones de sello hermético, a los medios a incubarse en O_2 1% se les hizó circular N_2 y posteriormente una mezcla N_2:O_2 (99:1).

[b]Expresada como pmol de MU producidos por 1 min a 37°C por 106 UFC.

[c]Cepa receptora UAP 5541; pRG960SD lleva al gene *gusA* sin promotor; pRGH562 tiene insertado al promotor de *nifH* de *A. diazotrophicus* después del Shine Dalgarno de *gusA* del pRG960SD; pRGH561 lleva un promotor con la "leader sequence" del virus AMV y el doble promotor del gene 35S del virus CaMV.

Los tallos de caña de azúcar de las variedades Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14 y RB 76-5418, inoculados con la cepa de *A. diazotrophicus* UAP 5541/pRGH562, que porta la construcción con la fusión promotor *nifH* de *A. diazotrophicus* con el gene reportero *gusA* fueron cultivados en invernadero con dos dosis de nitrógeno, una de 11 y la otra de 0.56 mmol de NH_4NO_3 por planta, aplicadas cada dos semanas. En ensayos histoquímicos de ß-glucuronidasa con X-Gluc no fue posible detectar actividad GUS en cortes de plantas (dos cortes por planta, 70 plantas), cultivadas en las condiciones de fertilización nitrogenada utilizadas.

III. BIODIVERSIDAD Y NUEVOS TAXAS EN LA FAMILIA ACETOBACTERACEAE

El conocimiento que se tiene de la diversidad de las poblaciones de *A. diazotrophicus* se ha ampliado con los aislamientos a partir de ambientes que no habían sido estudiados. En el caso de la planta y del suelo de café se han encontrado genotipos de esta bacteria que hasta ahora se han detectado exclusivamente en la planta de café y su rizósfera (Jiménez-Salgado y col., 1997, [Anexo III], Tabla 2). Del ambiente de la planta de café, además de las poblaciones de *A. diazotrophicus*, se han aislado otras cepas acéticas fijadoras de nitrógeno de un particular interés taxonómico, ya que éstas muestran algunas características fenotípicas distintas de las de *A. diazotrophicus* (Jiménez-Salgado y col., 1997, [Anexo III]), la única especie fijadora de nitrógeno descrita en la familia Acetobacteraceae (Gillis y col., 1989, Sievers y col., 1994).

Tabla 2. Frecuencia de genotipos de *Acetobacter diazotrophicus* aislados de distintos ambientes[a,b]

	procedencia									
Genotipo	caña de azúcar	rizoplano de caña	chiche harinosa	camote dulce	*Pennissetum purpureum*	hongo vesicular	cafeto	rizósfera de café		fuente[c]
ET1	0.425	nd	0.134	0.049	nd	nd	0.012	0.012	0.658[d]	(1, 2, 3)
ET2	0.037	nd	0.024	nd	nd	nd	nd	nd	0.061	(1, 2, 3)
ET3	0.085	0.012	nd	nd	0.012	0.012	nd	nd	0.021	(1, 2)
ET4	0.024	nd	nd	nd	nd	nd	nd	nd	0.024	(1, 2)
ET5	0.012	nd	nd	nd	nd	nd	nd	nd	0.012	(2)
ET6	nd	0.012	0.012	nd	nd	nd	nd	nd	0.024	(2)
ET7	0.049	nd	nd	nd	0.024	nd	nd	nd	0.073	(2)
ET8	nd	nd	nd	nd	nd	nd	0.012	0.012	0.024	(3)
ET9	nd	nd	nd	nd	nd	nd	0.012	nd	0.012	(3)
ET10	nd	nd	nd	nd	nd	nd	nd	0.012	0.012	(3)
ET11	nd	nd	nd	nd	nd	nd	0.012	nd	0.012	(3)
ET12	nd	nd	nd	nd	nd	nd	nd	0.012	0.012	(3)
ET14	nd	nd	nd	nd	nd	nd	nd	0.012	0.012	(3)

[a]frecuencia relativade genotipos de *A. diazotrophicus* en cada tipo de ambiente. Cada determinación indica la frecuencia respecto al total absoluto de aislamientos.
[b]nd indica que no se han aislado del genotipo en ese ambiente.
[c]fuente: 1, Caballero-Mellado y Martínez-Romero, 1994; 2, Caballero-Mellado y col., 1995; 3, Jiménez-Salgado y col., 1997.
[d]sumatoria de las frecuencias del genotipo.

Con base en la comparación filogenética de las secuencia nucleotídicas del gene ribosomal de la subunidad pequeña (SSU rDNA), las bacterias pertenecientes a la familia Acetobacteraceae forman los grupos: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Asaia* y *Gluconacetobacter* (Fig. 2). Uno de éstos está formado por la bacteria fijadora de nitrógeno *A. diazotrophicus*, y por los siguientes no fijadores de nitrógeno: *Gluconacetobacter liquefaciens* y la especie recientemente descrita *Gl. sacchari* (Franke y col., 1999; Yamada y col., 1997). Entre las secuencias de los genes SSU rDNA de estas especies se presentan similaridades mayores a 97.13% (Tab. 3; Franke y col., 1999; Fuentes-Ramírez y col., en prensa, [Anexo IV]).

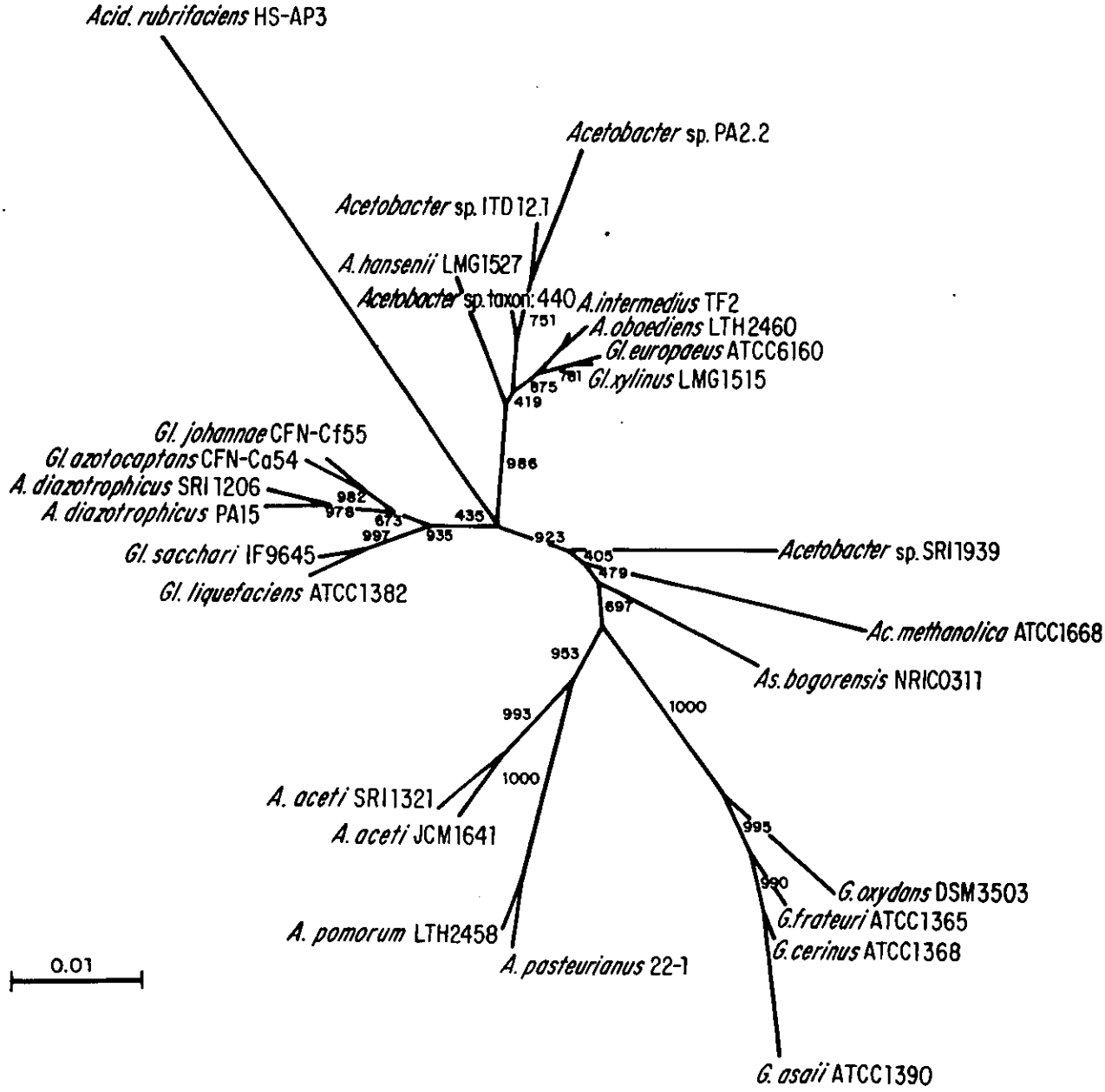


Figura 2. Árbol filogenético de la familia Acetobacteraceae. Las agrupaciones se hicieron de acuerdo a las distancias entre las secuencias del gene 16S. La secuencia de *Acidosphaera rubrifaciens* se incluyo como secuencia ajena al grupo. Abreviaturas: *Acid.*, *Acidosphaera*; *A.*, *Acetobacter*; *Gl.*, Gluconacetobacter; *Ac.*, *Acidomonas*; *As.*, *Asaia*; *G.*, *Gluconobacter*. Los números indican el valor de "boostrapp" de un total de 1000 árboles.

Algunos aislamientos de acetobacterias fijadoras de nitrógeno del ambiente rizosférico de la planta de café, mostraron una distancia genética considerable de *A. diazotrophicus* (Jiménez-Salgado y col., 1997, [Anexo III]). Los aislamientos de bacterias del café genéticamente distantes de *A. diazotrophicus* forman tres grupos denominados como SAd, DOR y APL. Decidimos determinar la ubicación taxonómica de los aislamientos del café, contemplando la posibilidad de contribuir al conocimiento de la diversidad de la familia y de las bacterias fijadoras de nitrógeno. La determinación de la diversidad taxonómica de cepas de Acetobacterias fijadoras de nitrógeno que se desarrollan en el ambiente rizosférico y endófito de la planta de café y que difieren de *A. diazotrophicus* (meta III) es abordado en el capítulo titulado "Polyphasic taxonomy of nitrogen-fixing acetic bacteria isolated from the rhizosphere of coffee plants", por Caballero-Mellado, J., Jiménez-Salgado, T., Tapia-Hernández, A., Wang, E. T., Martínez-Romero, E. y Fuentes-Ramírez, L. E., del libro "Highlights of Nitrogen Fixation Research" (Anexo V) y en el manuscrito titulado: "Novel nitrogen-fixing acetic bacteria, *Gluconacetobacter johannae* sp. nov., and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants", por Fuentes-Ramírez, L. E., Bustillos-Cristales, R., Tapia-Hernández, A., Jiménez-Salgado, T., Wang, E. –T., Martínez-Romero, E. y Caballero-Mellado, J. (Anexo VI). El citado manuscrito actualmente se encuentra en etapa de revisión en el International Journal of Systematic and Evolutionary Microbiology.

Se determinó trabajar inicialmente con una cepa del grupo SAd y una del grupo DOR. Utilizando los oligonucleótidos fD1 y rD1 (Weisburg, 1991), amplifiqué el gene 16S de las cepas de la rizósfera de café CFN-Ca54 (SAd) y CFN-Cf55 (DOR). Para la amplificación se utilizó la polimerasa Pwo. El producto de PCR fue clonado en el vector pCAPs, digerido previamente con la endonucleasa *Mlu*NI. Los fragmentos para la obtención de la secuencia nucleotídica se obtuvieron a partir del producto de PCR clonado y se ligaron en el vector pUC19. Las secuencias obtenidas se depositaron en

el GenBank bajo los números de acceso AF192761 y AF111841, para la cepa CFN-Ca54 y CFN-Cf55, respectivamente. Los grupos SAd y DOR podrían ser consideradas como nuevas especies, considerando las diferencias en secuencia con respecto a la especie más cercana, 97.89 y 97.96%, respectivamente [Tab. 3; Fuentes-Ramírez y col. Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov., and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int. J. Sys. Evol. Microbiol. (enviado, Anexo VI)].

Tabla 3. Matriz de similaridad de secuencias del gene ribosomal 16S de las especies de la familia Acetobacteraceae del grupo cercano a las nuevas especies fijadoras de nitrógeno.

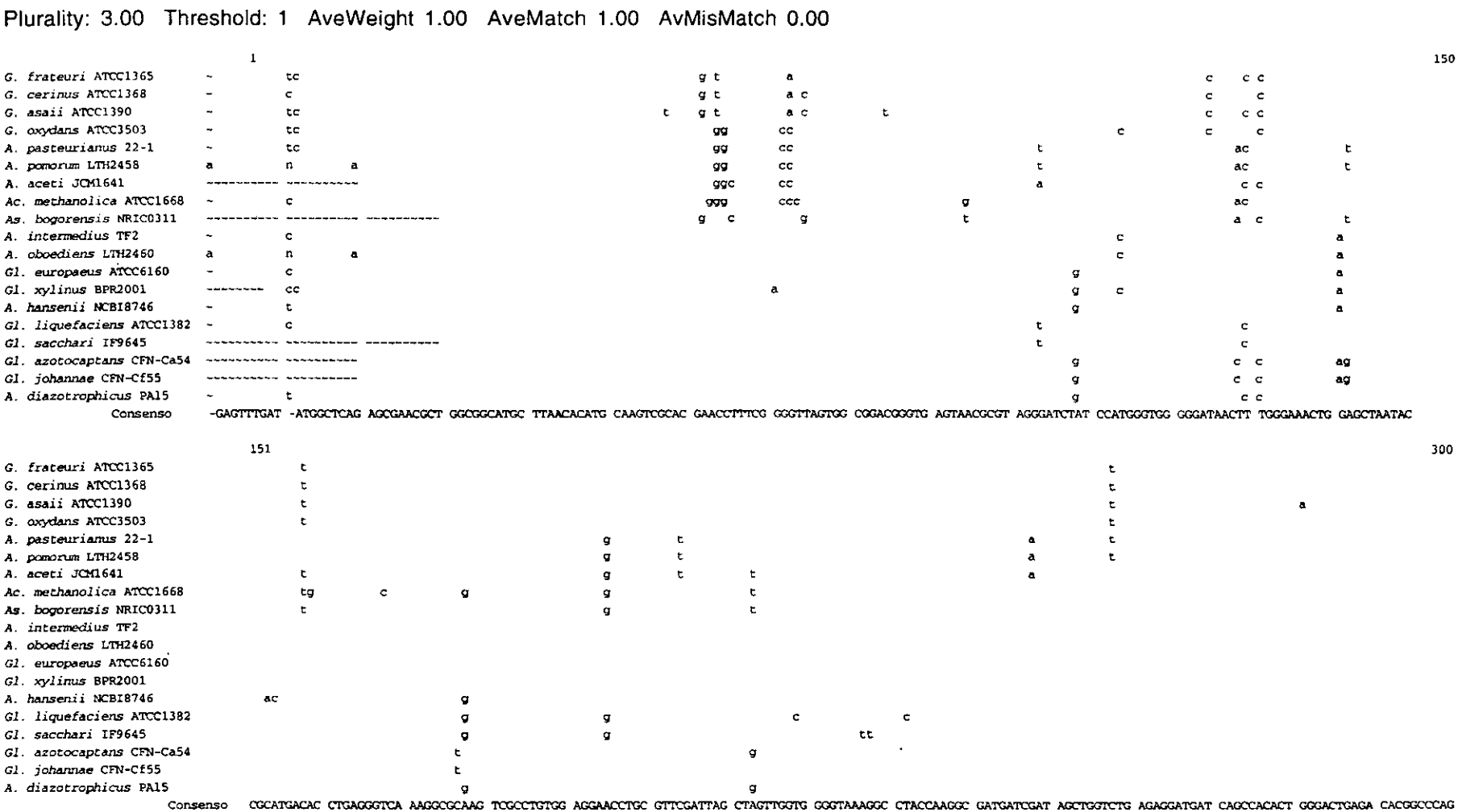
cepa	No. de Acceso	clave	2	3	4	5	6	7	8	9	10	11	12	
Gl. liquefaciens LGM1382	X5617	1	1.0000	0.9986	0.9930	0.9929	0.9930	0.9921	0.9909	0.9920	0.9922	0.9920	0.9875	1
Gl. liquefaciens SRI1244	AF127391	2		0.9914	0.9900	0.9800	0.9886	0.9871	0.9929	0.9883	0.9907	0.9920	0.9921	2
Gl. liquefaciens SRI1957	AF127393	3			0.9916	0.9915	0.9916	0.9785	0.9796	0.9796	0.9801	0.9834	0.9874	3
Gl. sacchari IF2-6	AF127412	4				1.0000	1.0000	0.9914	0.9902	0.9927	0.9929	0.9964	0.9944	4
Gl. sacchari IF9645	AF127413	5					1.0000	0.9813	0.9901	0.9825	0.9829	0.9863	0.9972	5
Gl. sacchari IF9701	AF127411	6						0.9899	0.9888	0.9913	0.9915	0.9949	0.9958	6
Gl. sacchari SRI1216	AF127406	7							1.0000	1.0000	0.9986	0.9993	0.9986	7
Gl. sacchari SRI1794	AF127407	8								1.0000	1.0000	0.9993	0.9972	8
Gl. sacchari SRI1953	AF127410	9									1.0000	0.9949	1.0000	9
Gl. sacchari SRI1853	AF127408	10										0.9993	0.9993	10
Gl. sacchari SRI1255	AF127409	11											0.9993	11
Gl. sacchari SRI1951	AF127404	12												
Gl. sacchari SRI1230	AF127405	13												
Gl. azotocaptans CFN-Ca54	AF192761	14												
Gl. johanae CFN-Cf55	AF111841	15												
A. diazotrophicus SRI1205	AF127402	16												
A. diazotrophicus SRI1206	AF127401	17												
A. diazotrophicus PAl5	X75618	18												
A. diazotrophicus SRI1212	AF127397	19												
A. diazotrophicus SRI1990	AF127392	20												
A. diazotrophicus-like SRI1941	AF127400	21												

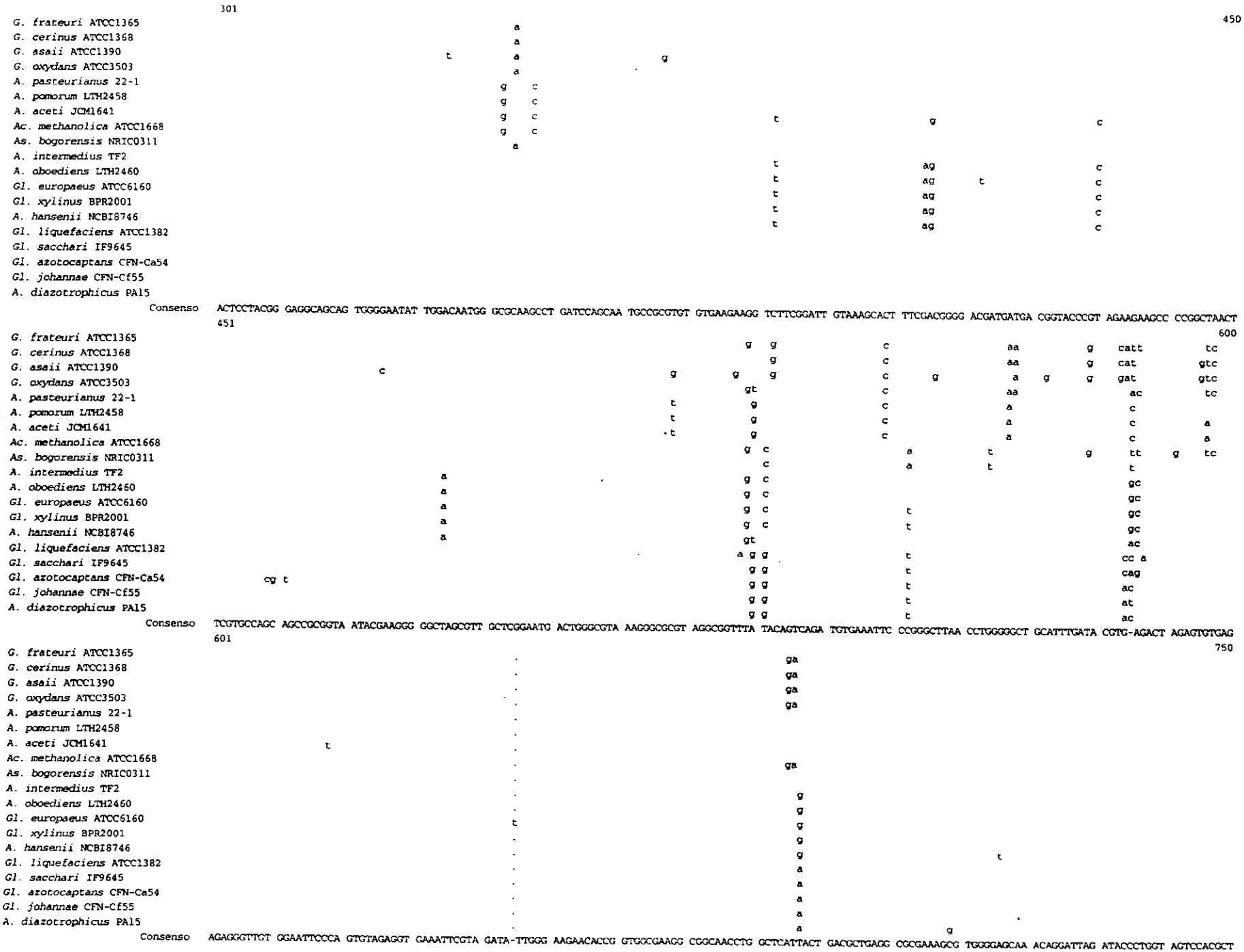
			13	14	15	16	17	18	19	20	21
Gl. liquefaciens LGM1382	X5617	1	0.9916	0.9744	0.9772	0.9813	0.9828	0.9818	0.9826	0.9851	0.9787
Gl. liquefaciens SRI1244	AF127391	2	0.9914	0.9714	9.9722	0.9813	0.9828	0.9829	0.9798	0.9821	0.9706
Gl. liquefaciens SRI1957	AF127393	3	0.9860	0.9789	0.9796	0.9813	0.9828	0.9817	0.9812	0.9732	0.9787
Gl. sacchari IF2-6	AF127412	4	0.9965	0.9749	0.9763	0.9821	0.9836	0.9846	0.9841	0.9806	0.9815
Gl. sacchari IF9645	AF127413	5	0.9965	0.9774	0.9788	0.9776	0.9791	0.9844	0.9841	0.9702	0.9808
Gl. sacchari IF9701	AF127411	6	0.9951	0.9762	0.9776	0.9821	0.9836	0.9846	0.9841	0.9791	0.9815
Gl. sacchari SRI1216	AF127406	7	0.9935	0.9655	0.9670	0.9821	0.9836	0.9828	0.9696	0.9873	0.9891
Gl. sacchari SRI1794	AF127407	8	0.9916	0.9650	0.9664	0.9821	0.9836	0.9818	0.9834	0.9873	0.9695
Gl. sacchari SRI1953	AF127410	9	0.9949	0.9687	0.9701	0.9828	0.9843	0.9847	0.9658	0.9873	0.9629
Gl. sacchari SRI1853	AF127408	10	0.9943	0.9672	0.9687	0.9821	0.9836	0.9829	0.9790	0.9873	0.9594
Gl. sacchari SRI1255	AF127409	11	0.9986	0.9718	0.9732	0.9813	0.9828	0.9834	0.9674	0.9828	0.9645
Gl. sacchari SRI1951	AF127404	12	0.9986	0.9632	0.9646	0.9821	0.9836	0.9785	0.9834	0.9873	0.9773
Gl. sacchari SRI1230	AF127405	13		0.9713	0.9727	0.9821	0.9836	0.9832	0.9834	0.9828	0.9759
Gl. azotocaptans CFN-Ca54	AF192761	14			0.9917	0.9806	0.9821	0.9827	0.9855	0.9754	0.9709
Gl. johanae CFN-Cf55	AF111841	15				0.9836	0.9851	0.9848	0.9855	0.9754	0.9730
A. diazotrophicus SRI1205	AF127402	16					0.9993	0.9910	0.9843	0.9821	0.9671
A. diazotrophicus SRI1206	AF127401	17						0.9925	0.9851	0.9836	0.9686
A. diazotrophicus PAI5	X75618	18							0.9986	0.9970	0.9752
A. diazotrophicus SRI1212	AF127397	19								0.9884	0.9732
A. diazotrophicus SRI1990	AF127392	20									0.9582

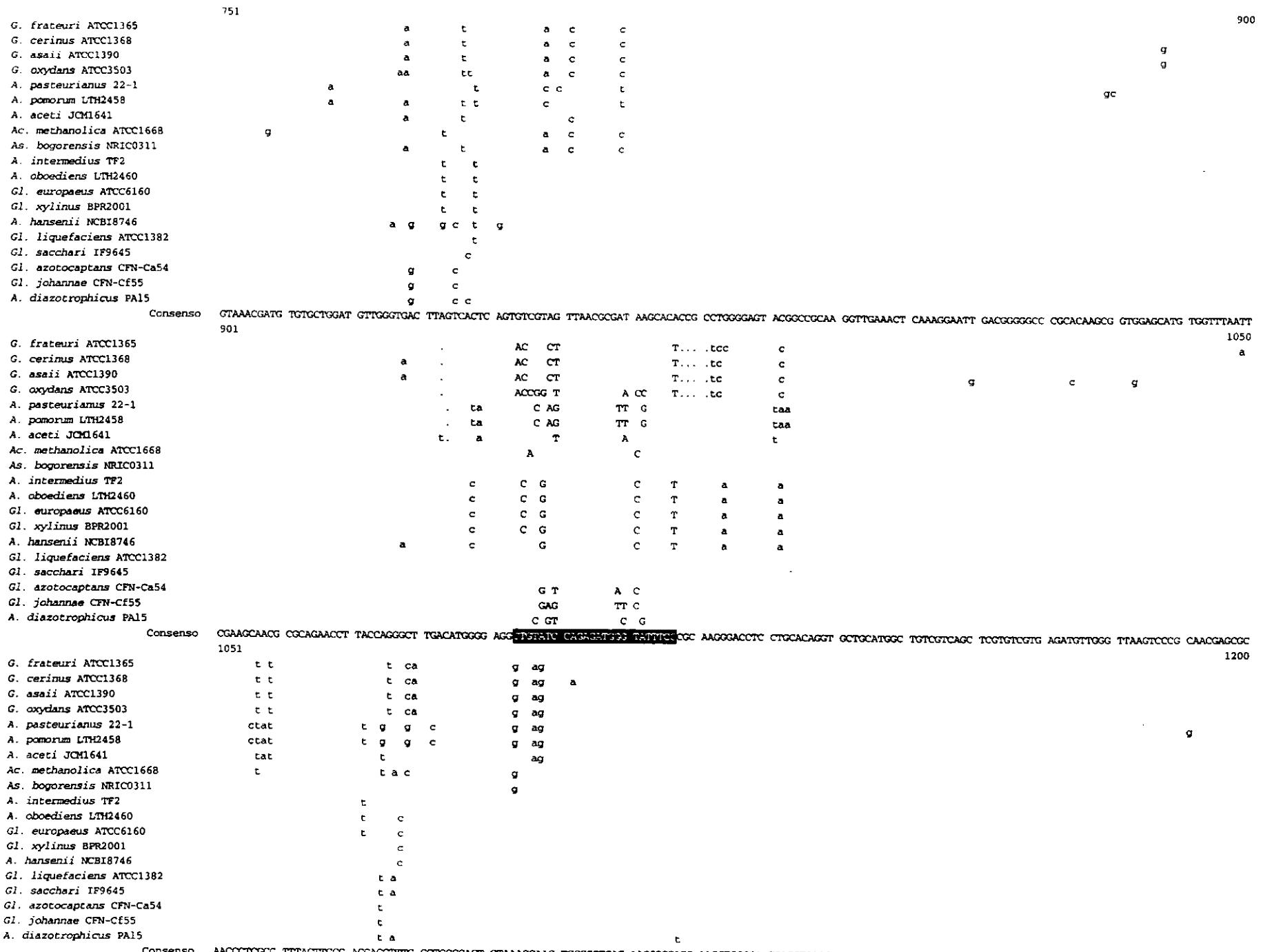
IV. USO DE OLIGONUCLEÓTIDOS ESPECÍFICOS PARA IDENTIFICACIÓN DE ACETOBACTERIAS FIJADORAS DE NITRÓGENO Y DE CUANTIFICACIÓN DE CÉLULAS

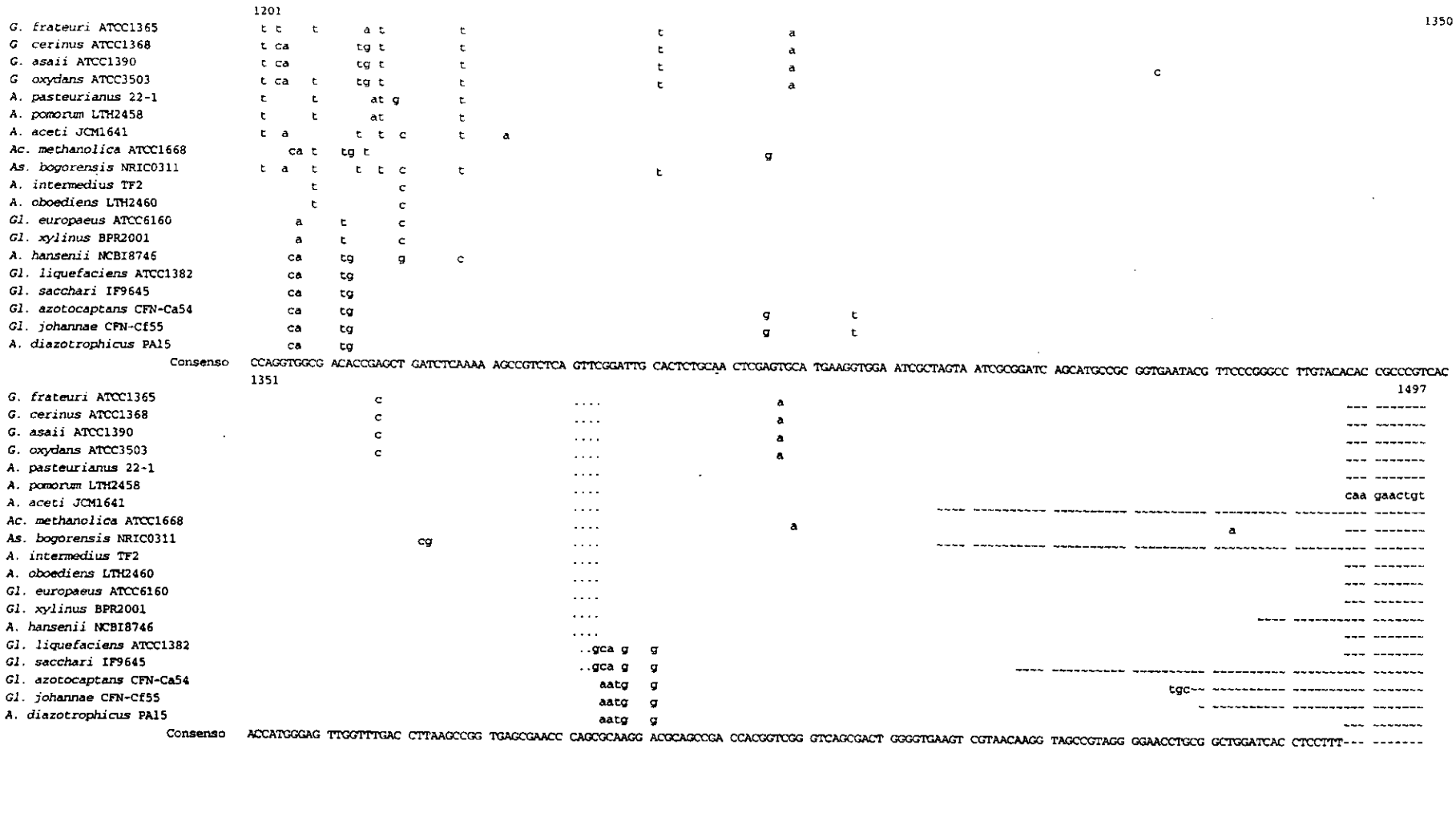
Se alinearon las secuencias de genes ribosomales de la subunidad pequeña de especies de la familia Acetobacteraceae disponibles en bancos (Fig. 3). Una región que corresponde a la numeración 989 a la 1042 de *E. coli* mostró la mayor divergencia entre las secuencias del gene 16S de la familia Acetobacteraceae. Dentro de esta misma zona se localizó una región que mostraba divergencia entre las cepas de *A. diazotrophicus*, SAd y DOR. A partir de esta región se diseñaron oligonucleótidos con potencial de ampliación específica por PCR (secuencia en Tabla 4). Utilizando una región conservada entre las secuencias del gene 16S de la familia Acetobacteraceae se diseñó un oligonucleótido (U475) universal para este grupo bacteriano (secuencia en Tabla 4). Bajo las condiciones citadas en la Tabla 5, el uso del oligonucleótido universal y de los oligonucleótidos denominados L925Ad, L927Gj y L923Ga produjeron amplificación específica de la especie *A. diazotrophicus* y de las cepas de los grupos SAd y DOR, respectivamente (Fig. 4; Fuentes-Ramírez y col., en prensa, [Anexo IV]).

Fig. 3. Alineamiento de secuencias del gene ribosomal 16S de las especies de la familia Acetobacteraceae[a].




301
450
G. frateuri ATCC1365
G. cerinus ATCC1368
G. asaii ATCC1390
G. oxydans ATCC3503
A. pasteurianus 22-1
A. pomorum LTH2458
A. aceti JCM1641
Ac. methanolica ATCC1668
As. bogorensis NRIC0311
A. intermedius TF2
A. oboediens LTH2460
Gl. europaeus ATCC6160
Gl. xylinus BPR2001
A. hansenii NCBI8746
Gl. liquefaciens ATCC1382
Gl. sacchari IF9645
Gl. azotocaptans CFN-Ca54
Gl. johannae CFN-Cf55
A. diazotrophicus PA15
Consenso ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGCAAGCCT GATCCAGCAA TGCCGCGTGT GTGAAGAAGG TCTTCGGATT GTAAAGCACT TTCGACGGGG ACGATGATGA CGGTACCCGT AGAAGAAGCC CCGGCTAACT
451
600
TCGTGCCAGC AGCCGCGGTA ATACGAAGGG GGCTAGCGTT GCTCGGAATG ACTGGGCGTA AAGGGCGCGT AGGCGGTTTA TACAGTCAGA TGTGAAATTC CCGGGCTTAA CCTGGGGGCT GCATTTGATA CGTG-AGACT AGAGTGTGAG
601
750
AGAGGGTTGT GGAATTCCCA GTGTAGAGGT GAAATTCGTA GATA-TTGGG AAGAACACCG GTGGCGAAGG CGGCAACCTG GCTCATTACT GACGCTGAGG CGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCT


751
900
G. frateuri ATCC1365
G. cerinus ATCC1368
G. asaii ATCC1390
G. oxydans ATCC3503
A. pasteurianus 22-1
A. pomorum LTH2458
A. aceti JCM1641
Ac. methanolica ATCC1668
As. bogorensis NRIC0311
A. intermedius TF2
A. oboediens LTH2460
Gl. europaeus ATCC6160
Gl. xylinus BPR2001
A. hansenii NCBI8746
Gl. liquefaciens ATCC1382
Gl. sacchari IF9645
Gl. azotocaptans CFN-Ca54
Gl. johannae CFN-Cf55
A. diazotrophicus PA15
Consenso GTAAACGATG TGTGCTGGAT GTTGGGTGAC TTAGTCACTC AGTGTCGTAG TTAACGCGAT AAGCACACCG CCTGGGGAGT ACGGCCGCAA GGTTGAAACT CAAAGGAATT GACGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT
901
1050
1051
1200
Consenso AACCCTCGCC TTTAGTTGCC AGCACGTTTG GGTGGGCACT CTAAAGGAAC TGCCGGTGAC AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTATGTCC TGGGCTACAC ACGTGCTACA ATGGCGGTGA CAGTGGGAAG



[a]La región de divergencia a partir de la que se diseñaron los oligonucleótidos específicos se muestra en tono invertido.

Tabla 4. Secuencia de los oligonucleótidos universal y específicos, utilizados en la identificación de las acetobacterias fijadoras de nitrógeno[a].

oligonucleótido[b]	especie[c]	secuencia nucleotídica
U475		5'-AATGACTGGGCGTAAAG-3'
L925Ad	*A. diazotrophicus*	5'-CAGCCATCTCTGACTG-3'
L923Ga	*Gl. azotocaptans*	5'-AATGCTCATCTCTGAACA-3'
L927Gj	*Gl. johannae*	5'-GAAATGAACATCTCTGCT-3'

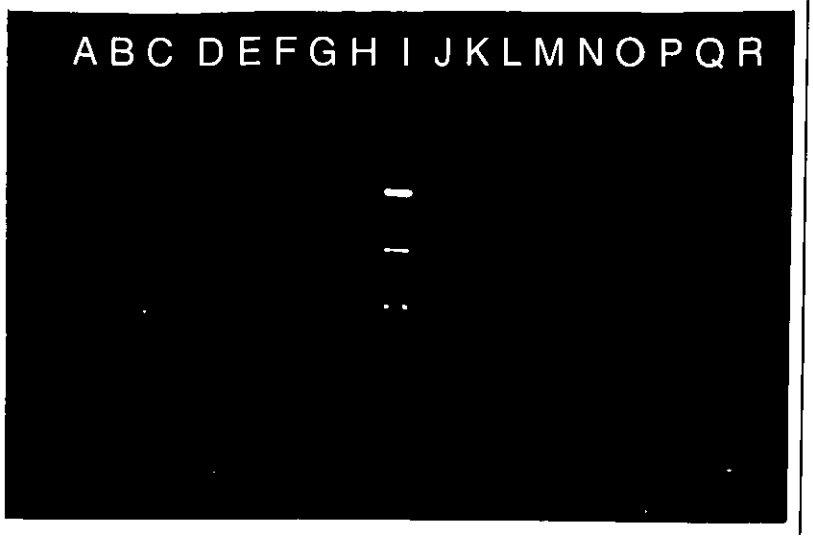
[a]Los productos esperados de ca. 400 bp se obtienen tanto a partir de ADN purificado como de células previamente lisadas por calor (95°C, 10 min).
[b]El oligonucleótido universal utilizado para las tres especies es el U475.
[c]El género *Gluconacetobacter* se abrevia como *Gl.* para evitar confusión con el género *Gluconobacter*.

Tabla 5. Condiciones de reacción de PCR para la obtención de productos específicos.

	desnaturalización			amplificación			elongación final		
oligonucleótidos[a]	ciclos	temp.[b]	tiempo[c]	ciclos	temp.	tiempo	ciclos	temp.	tiempo
	1	95	3		94	1	1	72	3
L925Ad				32	67	1			
					72	1			
	1	95	3		94	1	1	72	3
L923Ga				32	66	1			
					72	1			
	1	95	3		94	1	1	72	3
L927Gj				32	61	1			
					72	1			

[a]El oligonucleótido universal utilizado para las tres reacciones es el U475.
[b]Se expresa en grados centígrados.
[c]Se expresa en minutos.

a



b

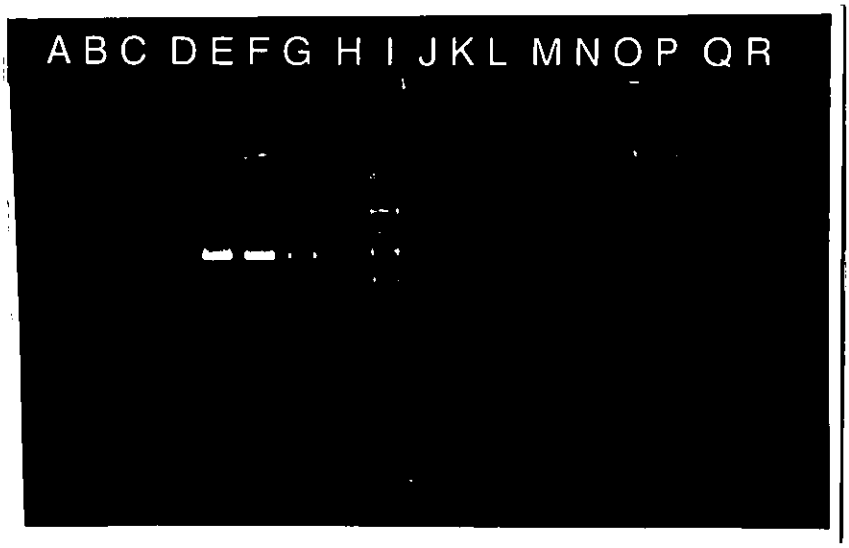


Figura 4. Productos obtenidos por amplificación, utilizando los oligonucleótidos específicos. Paneles: a) primers U475-L925Ad, b) U475-L923Ga, c) U475-L927Gj. Carriles: A-D, *A. diazotrophicus*; E-G, *Gl. azotocaptans*; H, J-L, *Gl. johannae*. Cepas: A, UAP5560; B, PAl5; C, UAP-Ac10; CFN-Cf56; E, CFN-Ca54; F, UAP-Ca97; G, UAP-Ca99; H, CFN-Cf55; J, UAP-Cf57; K, UAP-Cf76; L, CFN-Cf75; M, *Acetobacter* sp. Cf59; N, *A. hansenii* ATCC35959; O, *Gl. liquefaciens* ATCC14835; *G. oxydans* ATCC 19357; *Gl. xylinus*; ATCC 178; *G. asaii* ATCC 781. I, marcador de peso de 100-1200 pb. Las abreviaturas *G.* y *Gl.* significan *Gluconobacter* y *Gluconacetobacter*, respectivamente.

c

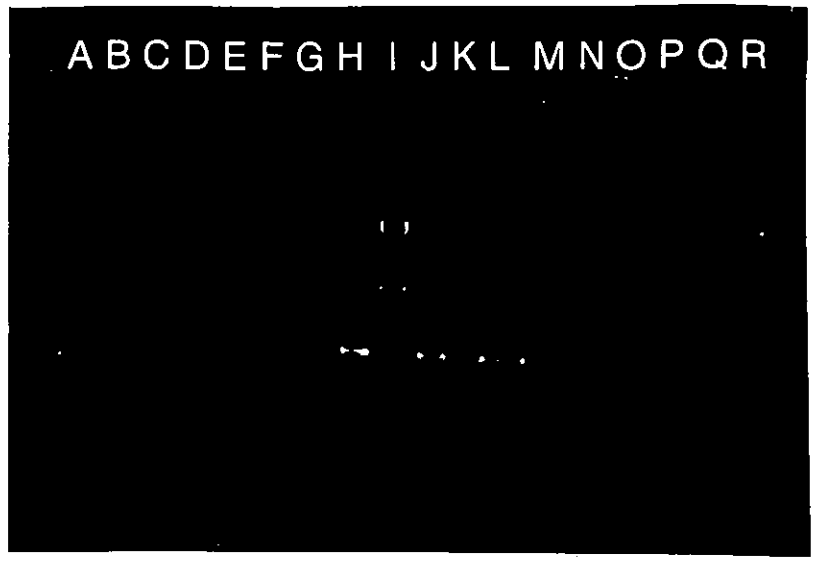


Figura 4. Productos obtenidos por amplificación, utilizando los oligonucleótidos específicos. Panel c) U475-L927Gj. Carriles: A-D, *A. diazotrophicus*; E-G, *Gl. azotocaptans*; H, J-L, *Gl. johannae*. Cepas: A, UAP5560; B, PAI5; C, UAP-Ac10; CFN-Cf56; E, CFN-Ca54; F, UAP-Ca97; G, UAP-Ca99; H, CFN-Cf55; J, UAP-Cf57; K, UAP-Cf76; L, CFN-Cf75; M, *Acetobacter* sp. Cf59; N, *A. hansenii* ATCC35959; O, *Gl. liquefaciens* ATCC14835; *G. oxydans* ATCC 19357; *Gl. xylinus*; ATCC 178; *G. asaii* ATCC 781. I, marcador de peso de 100-1200 pb. Las abreviaturas *G.* y *Gl.* significan *Gluconobacter* y *Gluconacetobacter*, respectivamente.

Los oligonucleótidos U475 y L925Ad fueron utilizados para la detección de células de *A. diazotrophicus*, junto con un ensayo tradicional de cuenta por método del número más probable (NMP) en plantas de caña de azúcar previamente inoculadas y que procedían de cultivo de tejidos. A partir de tubos de LGI inoculados con diluciones de planta (tres tubos por dilución) e incubados por 4 días a 30°C, alícuotas de 3 µl fueron mantenidas a 95°C por 10 min en una solución de Tween 20 al 0.1%. Posteriormente se añadieron $MgCl_2$ 2.5 mM, dNTPs 0.1 mM, 50 mM KCl, Tris-HCl 20 mM (pH 8.4), ambos oligonucleótidos a una concentración 20 nM, y 0.95 u de polimerasa Taq, en un volumen final de 15 µl. Las condiciones de reacción de PCR fueron las citadas en la Tabla 5. Los productos de reación se corrieron en elctroforesis en geles de agarosa. Con el uso de tablas de número más probable se obtuvo la cuenta de unidades bacterianas a partir del número de reacciones en las que se observó el producto. Esta cuenta se comparó con la cuenta obtenida por número más

probable obtenido a partir del número de tubos con LGI que mostraron crecimiento de *A. diazotrophicus*. La detección de células de *A. diazotrophicus* por PCR no varió en comparación a la deteccipon por NMP. Sin embargo, el tiempo de deteción por PCR se redujo al menos 3 días en comparación al tiempo necesarion para cuenta por NMP tradicional.

Así mismo, se intentó cuantificar células de *A. diazotrophicus* en cultivo, sin incubar previamente en medio de enriquecimiento. Se han probado distintas condiciones de reacción de PCR, sin embargo el desempeño de este procedimiento ha sido inferior (aproximadamente un orden de magnitud) a la cuantificación tradicional y la cuantificación mixta de PCR y método tradicional.

DISCUSIÓN

I. ECOLOGÍA DE LA COLONIZACIÓN

En experimentos de colonización, detectamos diferencias drásticas en la abundancia de *A. diazotrophicus* en plantas de caña de azúcar. Las diferencias poblacionales estuvieron ligadas a la cantidad de fertilizante nitrogenado aplicado, de tal manera que en las plantas más fertilizadas se encontraron poblaciones muy escasas. En ensayos *in vitro* en presencia de NH_4^+ se observó promoción de la multiplicación de *A. diazotrophicus* aún a concentración 10 mM por lo que se sugiere que los efectos de la fertilización observados no se deban a efectos directos del amonio sobre las bacterias y que tal vez como resultado de la fertilización nitrogenada la planta podría presentar cambios que afecten la colonización bacteriana. Se han documentado cambios en la concentración de sacarosa en relación a la fertilización nitrogenada de las plantas. Dado que los efectos reportados sobre la concentración sacarosa varían dependiendo del cultivar de planta empleado sería interesante cuantificar esta azúcar en las variedades que utilizamos, con y sin fertilizante.

El impacto observado de la fertilización nitrogenada sobre la colonización de *A. diazotrophicus* en caña de azúcar podría ser un ejemplo del tipo de efectos observados en otras asociaciones con bacterias diazotróficas. Kirchhof y col. (1997a) reportan una disminución en el número de células de fijadores de nitrógeno (géneros *Azospirillum* y *Herbaspirillum*) que colonizaron plantas de *Miscanthus sinensis*, *M. sacchariflorus* y *Spartina pectinata* que habían sido fertilizadas con nitrógeno, en comparación con plantas no fertilizadas.

En plantas de caña de azúcar inoculadas con la cepa UAP 5541/pRGS561 con expresión constitutiva de β-glucuronidasa sólo se detectó actividad GUS en ensayos en ausencia de azida de sodio. Lo anterior podría indicar que *in vivo* la cantidad de bacterias establecidas en el interior de la caña era menor al límite de detección, 10^5

UFC (unidades formadoras de colonia) por cm^3 de tejido, y que durante el periodo de incubación del ensayo (24 h, 30°C) el número de células habría rebasado ese límite por las duplicaciones de las bacterias. De hecho, la población bacteriana que colonizó plantas fertilizadas con baja cantidad de nitrógeno, calculada por el método del número más probable usando medio de enriquecimiento, no sobrepasó de 10^4 UFC por cm^3.

A diferencia del trabajo presente en que reportamos sólo los números de *A. diazotrophicus* en el interior de la planta de caña de azúcar, en otros estudios se han mostrado los números correspondientes a esta bacteria en asociación con la planta, sin diferenciar entre la población endófita y la población externa a los tejidos de la planta (James y col., 1994; Reis y col., 1994). Además de ello, la diferencia entre las cantidades de células de *A. diazotrophicus* en la caña entre los trabajos citados y el nuestro probablemente se relaciona también con el proceso mismo de la inoculación. Así, las plantas utilizadas por James y col. (1994) y por Reis y col. (1994) fueron inoculadas inicialmente con una cantidad determinada de células, pero tanto las plantas como las bacterias se mantuvieron con una fuente externa de carbono, permitiendo la multiplicación bacteriana artificial y dando como resultado una inoculación permanente de la planta durante el experimento. En contraste, al sustrato de las plantas que utilizamos no le adicionamos fuente de carbono y por lo mismo las bacterias sólo se mantuvieron con las fuentes de carbono sintetizadas por la planta. Dong y col. (1994) reportaron la presencia de 1.1×10^4 UFC de *A. diazotrophicus* por ml de fluído, en plantas obtenidas a partir de propágulos de tallo no inoculados artificialmente. La cuantificación que realizaron se hizo en medio de cultivo inoculado con fluído de plantas. Nosotros detectamos 2.9×10^3 UFC de *A. diazotrophicus* por g de tejido fresco de tallo en plantas inoculadas de seis meses de edad. J. Muñoz Rojas y J. Caballero Mellado (com. personal) han detectado en plantas de caña de azúcar inoculadas con *A. diazotrophicus*, poblaciones de 10^5 UFC en el interior de raíz y hasta 10^3 UFC en la parte aerea de la planta, a los 35 días de la inoculación. Las diferencias

observadas entre las cantidades citadas por Dong y col. (1994) y las nuestras podrían esperarse por la variación que naturalmente se observa en la colonización de plantas por microorganismos asociados y que se relaciona con factores diversos tales como la edad de la planta (Mahaffee y Kloepper, 1996; McInroy y Kloepper, 1995; J. Muñoz Rojas y J. Caballero Mellado, com. personal) y la variedad de ésta (da Silva y col., 1995).

En la relación de *A. diazotrophicus* con la caña de azúcar el papel del hospedero parece ser determinante. Así, la concentración bacteriana en el interior de las plantas inoculadas no parece rebasar un límite determinado, no obstante que en ciertos sitios de colonización (apoplasto y tal vez floema) las fuentes de carbono (sacarosa, glucosa y fructosa) son abundantes. Tal vez la capacidad de la planta de restringir la proliferación de *A. diazotrophicus* previene que este crezca excesivamente convirtiéndose en un patógeno.

Por ensayo histoquímico de actividad GUS logramos detectar colonización de *A. diazotrophicus* en vasos de xilema de tallo, con lo que la propuesta de Dong y col. (1994 y 1997) sobre la imposibilidad de esta bacteria de ubicarse en este tejido en plantas sanas parece errónea bajo las condiciones usadas en el presente trabajo. Además observamos que *A. diazotrophicus* aparentemente también colonizó tejido de floema. Por observación microscópica, estos autores no encontraron células bacterianas en otras estructuras del vegetal y cuando introdujeron a *A. diazotrophicus* en el xilema detectaron la expresión de una severa reacción, probablemente defensiva, por parte de la planta (Dong y col., 1994 y 1997); concluyen que *A. diazotrophicus* se encuentra en los espacios intercelulares del tallo de la caña de azúcar y que no coloniza de manera natural a los conductos del xilema. Sin embargo, si la cantidad de *A. diazotrophicus* en plantas no inoculadas es tan baja como la que se presenta en plantas inoculadas, sería muy baja la posibilidad de detectar microscópicamente a esta bacteria en tejido de plantas adultas sin la ayuda de algún gene reportero o de

métodos inmunológicos. Por lo anterior, resulta improbable que en los trabajos de Dong y col. (1994 y 1997) hubieran observado células de *A. diazotrophicus* en secciones microscópicas realizadas aleatoriamente. Por otra parte ellos suponen que las estructuras detectadas por microscopía electrónica de transmisión (MET) y de barrido (MEB) en el interior de espacios intercelulares son *A. diazotrophicus* pero carecen de pruebas adicionales. Por añadidura, el aislamiento de *A. diazotrophicus* a partir de líquido apoplástico no excluye su presencia en el contenido de los conductos de xilema. Por último, en su reporte de 1997, el daño excesivo a la planta por las condiciones en que ellos realizaron el proceso de inoculación, y no la bacteria misma, podría muy bien ser la causa de la producción de respuesta de defensa de la planta. Dong y col. (1997) llevaron a cabo la inoculación de las plantas utilizando un medio que favorece la replicación de *A. diazotrophicus* y durante un tiempo mucho mayor que el de generación de la bacteria. En los experimentos reportados por Dong y col. (1997) tal vez la cantidad de células bacterianas introducidas en el xilema fue excesiva, o bien la planta presentó respuesta defensiva al daño físico ocasionado por el proceso de inoculación, al mantener al tallo en contacto con una solución con un pH demasiado bajo (probablemente ca. 2.5). La colonización de xilema de tallo, como se evidencia en nuestro trabajo, coincide con las observaciones de James y col. (1994). Nosotros además proponemos que el xilema no es el único tejido colonizado, sino que a través de él se da la migración de *A. diazotrophicus* hacia otros tejidos y regiones vegetales, tales como la corteza del tallo o los espacios intercelulares. Los conductos del xilema se desarrollan a partir de células en un proceso que a continuación se describe. Inicialmente en las paredes internas de las células originales se deposita pared secundaria, que se supone se encuentra más lignificada que la pared primaria. En una posterior actividad hidrolítica, se disuelven las paredes primarias poco lignificadas. Este último proceso daría lugar a que se formaran las perforaciones que existen entre tubos funcionales contiguos (Torrey y col., 1971). Estas perforaciones tienen casi el mismo

diámetro de la luz interna de los conductos, por lo que permitirían el paso de bacterias con facilidad. Nosotros sugerimos que *A. diazotrophicus*, que produce fitohormonas como las auxinas (Fuentes-Ramírez y col., 1993) podría inducir la diferenciación de los conductos del xilema a través de la liberación de hormonas. Dong y col. (1994) lograron aislar *A. diazotrophicus* a partir del fluído procedente del apoplasto de tallo de caña de azúcar. De manera similar, en el apoplasto de maíz y de teosinte (*Zea luxurians*) se han obtenido aislamientos del género diazotrófico *Klebsiella* (Palus y col., 1996). La ubicación de *A. diazotrophicus* tanto en el espacio intercelular, así como en el interior de tejido de floema y en el interior de células le podría proveer a la bacteria de una mayor disponibilidad de carbohidratos, en comparación al que obtendría del fluído de xilema de esta planta (Welbaum y Meinzer, 1990; Welbaum y col., 1992).

Distintas especies bacterianas, incluyendo endófitos y fitopatógenos, se han observado colonizando diferentes tejidos vegetales. El actinomiceto *Clavibacter xyli* subsp. *xyli*, agente etiológico del "ratoon stunting disease" de la caña de azúcar (Davis y col., 1984) coloniza el sistema vascular del xilema de la planta. La planta responde a la presencia del patógeno con la síntesis y liberación de polisacáridos en este tejido (Kao y Damann, 1980). La cepa BH72 de la bacteria diazotrófica *Azoarcus* sp. penetra la corteza de raíz del pasto Kallar (*Leptochloa fusca*, L.) y del arroz, colonizando regiones intra e intercelulares. Probablemente a partir de ahí se desplaza al xilema de raíz, vía que pudiera utilizar para dispersarse a otros tejidos de la planta (Hurek y col., 1994; Reinhold y Hurek, 1988). De plantas de arroz se aisló la cepa fijadora de nitrógeno A15 de *Pseudomonas stutzerii* (anteriormente *Alcaligenes fecalis*, Vermeiren y col., 1999). Se pudo observar que esta cepa coloniza los espacios intercelulares de la raíz de esa gramínea. También en la raíz la bacteria se detectó dentro de células que no mostraban daño aparente (You y Zhou, 1989). En cultivos celulares de arroz inoculados con *P. stutzerii* A15 los autores obtuvieron evidencia de transferencia de nitrógeno fijado utilizando $^{15}N_2$. En esta misma gramínea se ha observado inducción de

promoción en el crecimiento por inoculación de la cepa BH72 de *Azoarcus*. Sin embargo, aparentemente este efecto no tuvo relación con la fijación de nitrógeno (Hurek y col., 1994). En plántulas de trigo inoculadas con distintas cepas de cianobacterias se pudo determinar la colonización endófita de raíz, así como la actividad reductora de acetileno de una cepa del género *Nostoc* en la asociación con la planta (Gantar y col., 1991a; Gantar y col., 1991b). En un ensayo de inoculación de plantas de tabaco regeneradas *in vitro* con *Anabaena variabilis* se pudo determinar la colonización de distintos tejidos del tallo de las plántulas, a la vez que se presentaba actividad reductora de acetileno (Gusev y col., 1986).

A raíz del aislamiento de *A. diazotrophicus* en otros vegetales no considerados como hospederos, comenzamos el estudio de otros modelos posibles. Entre éstos probamos la posibilidad de asociación de *A. diazotrophicus* con la planta de arroz (var. Morelos A-92), una gramínea de crecimiento rápido. En este último ensayo pudimos aislar las cepas inoculadas a partir de plantas de 17 días de edad, también observamos una apreciable actividad GUS en las mismas plantas inoculadas con la cepa UAP 5541/pRGS561, con expresión constitutiva del gene reportero, aún incubando con azida de sodio, pero únicamente en regiones superficiales de la planta (resultados no mostrados). Sin embargo, en plantas de 40 días sólo se logró el aislamiento de las cepas, pero no se detectó actividad GUS en planta, en ensayo con azida de sodio. La presencia de las cepas inoculadas, junto con la inactividad GUS en las plantas de 40 días, a diferencia de lo que se detectó en plantas más jóvenes, apoyaría la hipótesis de la disminución del metabolismo bacteriano por la asociación con la planta después de cierta edad de la planta o por arriba de determinada concentración bacteriana. Los mecanismos mediante los cuales la planta regularía la actividad metabólica de las bacterias en su interior serían interesantes de investigarse.

II. GENES DE NITROGENASA Y SU EXPRESIÓN EN PLANTA

No pudimos detectar actividad de la fusión *nifH::gusA* en las plantas de caña de azúcar con la cepa correspondiente. La baja densidad de células bacterianas en la planta podría haber constituido un factor que impidiera la detección histoquímica de la expresión de la fusión *nifH::gusA*. Otra posibilidad que no excluye a la primera es que la actividad GUS de la bacteria no se haya podido detectar debido a una actividad bacteriana metabólica mínima dentro de la planta. Por otra parte, datos no publicados de inoculación de plantas de maíz con *A. diazotrophicus* (P. Estrada y J. Caballero, comunicación personal), indican que la población bacteriana se incrementa hasta un nivel determinado y posteriormente se estabiliza, a la vez que aparentemente se presenta una reducción en su metabolismo.

Debido a que no utilizamos cuantificación directa de fijación de nitrógeno, no podemos afirmar que no exista contribución de nitrógeno a la planta por parte de la bacteria. Por otro lado es necesario realizar ensayos que involucren a todo el ciclo de vida de la planta. Posiblemente sólo durante ciertos periodos en el ciclo de la planta se presente actividad bacteriana detectable en plantas con poblaciones de microorganismos diazótrofos.

Sevilla y col. (1998, y resultados sin publicar, [Núm. Acceso AF030414 y AF072689]) han obtenido la secuencia nucleotídica de dos amplios fragmentos de la región *nif* de *A. diazotrophicus* PAl5^T. Uno de los fragmentos tiene el orden *nifH nifD nifK* y *nifE*, todos en el mismo sentido y el otro fragmento contiene a *nifA nifB* y al orf *fdxN*. En *A. diazotrophicus* PAL5^T los genes *nifHDK* se presentan agrupados posiblemente en un operón, tal como se ha encontrado en bacterias fijadoras ubicadas en distintos grupos taxonómicos: *Clostridium cellulolyticum* (Bagnara y col., resultados sin publicar, [Núm. Acceso X60727]), *Frankia* (Specq y Normand, resultados sin publicar, [Núm. Acceso U53363]), *Fischerella* sp. (Luo y Stevens, resultados sin publicar, [Núm Acceso U49514]; Saville y col., 1987), *Plectonema* (Barnum y Gendel,

1985), *Cyanothece*, *Syneccochoccus* (Kallas y col., 1985) *Azorhizobium caulinodans* (Denèfle y col., 1987), *Azospirillum brasilense* (de Zamarockzy y col., 1989), *Rhizobium leguminosarum* (Krol y col., 1982), *Rhizobium etli* (Quinto y col., 1985), *Rhizobium meliloti* (Ruvkun y col., 1982), *Rhodobacter* (Avtges y col., 1983), *Herbaspirillum seropedicae* (Machado y col., 1996), *Thiobacillus ferrooxidans* (Pretorius y col., 1987; Rawlings, 1988), *Azotobacter vinelandii* (Brigle y col., 1985; Krol y col., 1982) y *Klebsiella pneumoniae* (Riedel y col.,1979).

En cuanto a la naturaleza de un posible gene codificado por el orf1 de *A. diazotrophicus* UAP 5560 probablemente no se trata de alguna ferredoxina ya que no posee los aminoácidos conservados entre las ferredoxinas (no se muestra). A 30 bases en dirección 3' después del orf1 se encuentra una secuencia invertida repetida (IR) que podría formar una estructura cruciforme sumamente estable. Esta IR pudiera tener función como terminador o como protector contra la degradación del mRNA, si el orf1 fuera trascrito, como se ha sugerido en el orf *fdxD* que se encuentra hacia la dirección 5' de *nifH* de *R. capsulatus* (Willison y col., 1993). En *A. brasilense* secuencias invertidas repetidas se encuentran bordeando a *nifH* (de Zamaroczy y col., 1989), los autores detectan dos transcritos de diferente tamaño y sugieren que en la producción del más pequeño podrían intervenir las IR localizadas entre *nifH* y *nifD*. de Zamaroczy y col. (1989) no relacionan las IR de arriba de *nifH* de *A. brasilense* con ningún orf. En las regiones reguladoras de genes de heat-shock de distintos organismos (citados en Wetzstein y col., 1992; Yuan y Wong, 1995), así como en las del gene de la superóxido dismutasa de *E. coli* (Takeda y Avila, 1986) y de *Lactococcus lactis* (Sanders y col., 1995) se han encontrado secuencias IR. En el operon *dnaK* de *B. subtilis* una IR se ubica entre el inicio de transcripción y el inicio del primer orf del operón (orf39), a 11 nt abajo de un promotor putativo tipo -10 -35 (Wezstein y col., 1992). En ese operón se demostró una función reguladora negativa de la IR que lo precede (Zuber y Schumann, 1994). En el operón *groESL* de *B. subtilis*

el promotor del tipo -10 -35 es sucedido por el sitio de inicio de transcripción y posteriormente por una IR (Schmidt y col., 1992). Introduciendo inserciones de distinto tamaño entre el inicio de transcripción y la IR se demostró el papel de la IR como operador en la transcripción del operón *groESL* de *B. subtilis* (Yuan y Wong, 1995), también en ese mismo trabajo se observó el papel de IR en la estabilidad del transcrito en cultivos bajo temperatura normal. La IR de *nifH* de *A. diazotrophicus* UAP 5560 se localiza a una distancia considerable del inicio de *nifH* por lo que no forma parte del transcrito de ese gene. El hecho de que esta IR se encuentre en una región muy diferente a las IR de los genes de choque térmico anteriormente descritos no excluye que pueda jugar algún papel en la regulación transcripcional de *nifH* tomando en cuenta que la IR está próxima al sitio putativo de unión al regulador positivo NifA (a 37 nt en dirección 5').

La formación del complejo abierto de la transcripción de *nifH* en *K. pneumoniae* y en *R. meliloti* requiere de la interacción de la polimerasa de RNA dependiente de σ^{54} y de la proteína NifA (Morett y Buck, 1989). Este evento es facilitado por la intervención de la proteína "Integration Host Factor" (IHF), (Santero y col., 1989). La localización de un probable sitio de reconocimiento de IHF inmediatamente arriba del promotor presuntivo -12 -24 de *nifH* de *A. diazotrophicus* UAP 5560 indica la posible intervención de esta proteína en la transcripción de *nifH*.

III. DIVERSIDAD Y NUEVOS TAXAS EN LA FAMILIA ACETOBACTERACEAE

La detección de nuevas asociaciones en las que participarían *A. diazotrophicus* y plantas con características ecofisiológicas distintas a las de la caña de azúcar, modifica sustancialmente el modelo que limitaba la posibilidad de asociación de esta bacteria sólo con plantas que mantuvieran reservas altamente concentradas de sacarosa. De igual manera, *A. diazotrophicus* había sido encontrado sólo esporádicamente en el suelo, por lo que también se excluía su capacidad de sobrevivir en dicho ambiente. En el interés por el estudio de la ecología de *A. diazotrophicus* encontramos a esta bacteria en la rizósfera y en tejidos de la planta de café (Jiménez-Salgado y col., 1997, [Anexo III]). El aislamiento de cepas pertenecientes a la especie *A. diazotrophicus* a partir de los sitios mencionados, modifica el modelo de asociación ya expuesto.

Teniendo como precedente los trabajos realizados por Caballero-Mellado y Martínez-Romero (1994), Caballero-Mellado y col. (1995), [Anexo II] y Jiménez-Salgado y col. (1997), [Anexo III], hemos colaborado en el análisis de la estructura poblacional de *A. diazotrophicus* utilizando nuevos aislamientos obtenidos a partir de caña de azúcar y de otras fuentes. Para distintas especies se ha postulado la relación existente entre las poblaciones y el medio que éstas habitan (Bidochka y col., 1997; Whittam y col., 1983). Con los aislamientos de *A. diazotrophicus* que han sido analizados podemos observar una aparentemente especificidad de ciertos genotipos por determinados habitats (Tabla 2). En la planta de café y su rizósfera hemos aislado genotipos que no han sido detectados en otros ambientes (ETs 8, 9, 10, 11, 12 y 14). Esta planta tiene características que son únicas en comparación a las demás, tales como el hecho de ser leñosa y probablemente con un diferente contenido de compuestos de reserva, así como de compuestos de metabolismo secundario. Lo anterior nos permite sugerir que posiblemente estos genotipos del café poseen adaptaciones que les permiten colonizar a esta planta. Por otra parte, si fuera cierta la

existencia de ecotipos en *A. diazotrophicus*, entonces resultaría clara la utilidad de la metodología de multilocus para su rápida y fácil detección. La caña de azúcar es con mucho el más explorado de los distintos ambientes de los que se ha intentado aislar *A. diazotrophicus*, por lo que aunque los ETs 4 y 5 han sido encontrados exclusivamente de manera endofítica en esta planta no podemos excluir que no se encuentren en otros ambientes menos estudiados. El análisis de parámetros poblacionales de los aislamientos de *A. diazotrophicus* obtenidos a partir de diversos ambientes ha mostrado que la estructura de esta bacteria es clonal (Caballero-Mellado y Martínez-Romero, 1994; Caballero-Mellado y col., 1995, [Anexo II]).

El análisis fenotípico y genotípico de aislamientos tipo SAd y DOR procedentes de la planta de café (Jiménez Salgado y col., 1997, [Anexo III]) mostró diferencias considerables entre ellas y también con respecto a *A. diazotrophicus* (Fuentes Ramírez y col., enviado, [Anexo VI]). Esas diferencias sugieren que esos aislamientos pertenecen especies de fijadoras de acetobacterias fijadoras de nitrógeno distintas entre ellas y también distintas de *A. diazotrophicus*. Debido a que por filogenia del gene 16S esas especies se ubican dentro del género ahora conocido como *Gluconacetobacter* (en adelante este género será abreviado como *Gl.* para evitar confusiones con el género *Gluconobacter*), las dos nuevas especies sugeridas se denominaron como *Gl. johannae* y *Gl. azotocaptans* (Fuentes Ramírez y col., enviado, [Anexo VI]).

IV. USO DE OLIGONUCLEÓTIDOS ESPECÍFICOS PARA IDENTIFICACIÓN DE ACETOBACTERIAS FIJADORAS DE NITRÓGENO Y DE CUANTIFICACIÓN DE CÉLULAS.

A partir de una región del gene ribosomal de la subunidad pequeña que muestra variabilidad en la familia Acetobacteraceae fue posible diseñar oligonucleótidos con utilidad taxonómica para *A. diazotrophicus*, así como para las especies propuestas *Gluconacetobacter johannae* (DOR) y *Gluconacetobacter azotocaptans* (SAd). Los oligonucleótidos específicos L925Ad, L923Ga y L927Gj en combinación con el oligonucleótido universal U475, permiten identificar cepas de las acetobacterias fijadoras de nitrógeno. Es conveniente señalar que el hecho de que un cierta característica o prueba útiles en la identificación taxonómica sean consideradas específicas no implica que en cualquier condición muestren este comportamiento. Esto es debido a que además de los organismos en los que se ha ensayado o partir de los que se ha diseñado una determinada prueba, muy probablemente en la naturaleza existirán otros organismos que presentarán comportamiento semejante respecto a esa prueba y que no necesariamente pertenecerán al grupo taxonómico de interés. A este respecto, Dykhuzen (1998) ha estimado en más de 10^9 especies de procariontes que podrían actualmente existir en la biósfera. En el grupo de organismos de nuestro trabajo, en dos trabajos previos se han sugerido dos oligonucleótidos específicos para la identificación de *A. diazotrophicus* (Kirchhof y col., 1997b; Sievers y col., 1998). El primer sugerido por Kirchhof y col. (1997b) se diseño para amplificar un fragmento del gene 23S y el de Sievers y col. (1998) para amplificar un fragmento del gene 16S, ambos de *A. diazotrophicus*. Sin embargo, por PCR de las especies *Gl. johannae* y *Gl. azotocaptans* utilizando por separado ambos oligonucleotidos junto con oligonucleótidos universales, hemos observado amplificación de producto de tamaño igual al de *A. diazotrophicus*.

Los oligonucleótidos específicos que proponemos se unen a una región que corresponde en *E. coli* al tallo 33 que tiene interacción con la proteína S19 (Brimacombe, 1995). Aunque esta región muestra variabilidad entre las cepas de acetobacterias fijadoras de nitrógeno, la secuencia permitiría mantener la estructura de tallo correspondiente (Fig. 5).

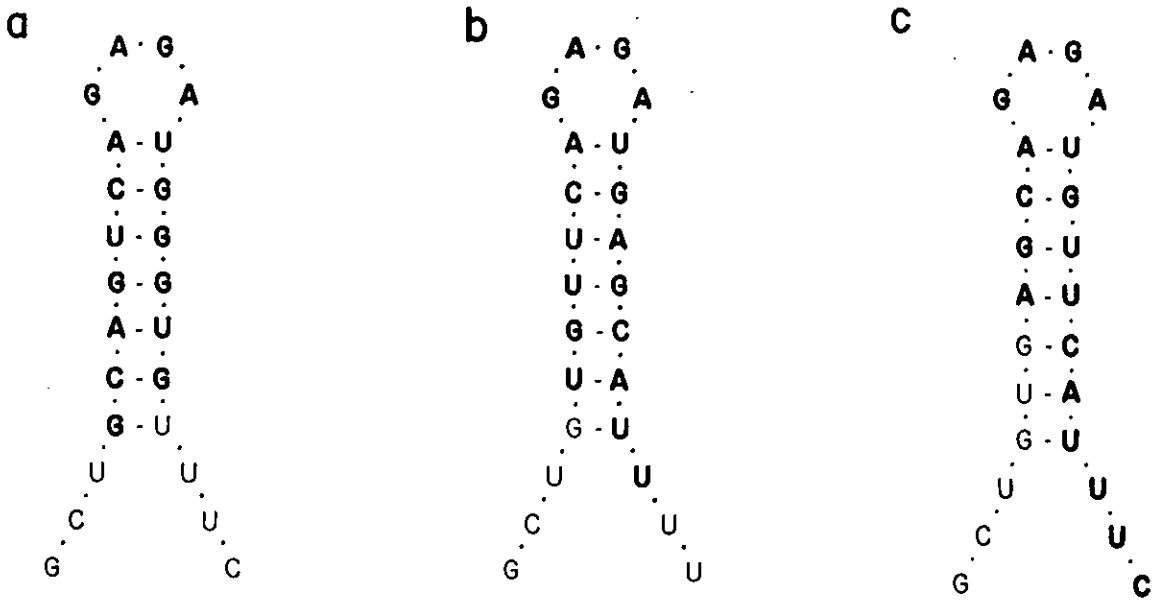


Figura 5. Formación de estructuras secundarias teóricas en las regiones variables del gene 16S de las acetobacterias fijadoras de nitrógeno. Las regiones corresponden al asa 33 de *E. coli* (Brimacombe, 1995). Paneles: a) *A. diazotrophicus* PAl5; b) *Gl. azotocaptans* CFN-Ca54; c) *Gl. johannae* CFN-Cf55. En negritas se indican las bases con las que se aparean los oligonucleótidos específicos.

La utilización de métodos combinados de número más probable y de PCR específico permitió tener un índice de la población de *A. diazotrophicus* en planta. La utilización de esta técnica redujó el tiempo necesario para la cuantificación de *A. diazotrophicus* en plantas gnotobióticas. La diversidad de organismos presentes en las plantas heteroxénicas demora la cuantificación de *A. diazotrophicus*, ya que se hacen necesarias varias resiembras de enriquecimiento. La utilización de la técnica mixta de NMP y PCR específico probablemente permita disminuir sensiblemente el tiempo utilizado en la cuantificación de *A. diazotrophicus* en plantas heteroxénicas.

También se intentó cuantificar células de *A. diazotrophicus* en cultivo utilizando PCR sin un periodo previo de enriquecimiento. Sin embargo, la cantidad determinada regularmente fue un orden de magnitud menor a la determinada por número más probable obtenido por crecimiento en medio de enriquecimiento o por la técnica mixta de enriquecimiento y PCR. La técnica de cuenta directa por PCR se ha realizado con otros microorganismos con resultados similares a los obtenidos en este trabajo, es decir una menor capacidad de detección por PCR que por otros métodos (Féray y col., 1999; Picard y col., 1992). En esos trabajos se sugirió que la menor eficiencia del uso de PCR respecto a otras técnicas podría deberse a la presencia de compuestos con actividad inhibitoria de la reacción de amplificación. En los ensayos realizados en esta tesis la presencia de sustancias con posible actividad inhibitoria no debería interferir ya que las reacciones se llevaron a cabo utilizando alícuotas procedentes de diluciones de cultivos. Posiblemente el problema en nuestros ensayos se relacione con el proceso de obtención del ADN, por lo que nuevas condiciones de este proceso serán probadas en futuro próximo.

Las asociaciones que se establecen entre bacterias endófitas y plantas constituyen modelos relativamente nuevos para la comprensión de las interacciones ecológicas y por lo tanto existe un gran desconocimiento sobre los mecanismos de su

interacción. En la asociación de *Herbaspirillum rubrisubalbicans* con caña de azúcar, el resultado final puede ser un balance entre el beneficio o el daño para la planta (Olivares y col., 1997; Oliveira y col., 1999). Probablemente las interacciones benéficas y "neutras", tales como las de *A. diazotrophicus*, sean el producto de la coevolución entre plantas y microorganismos patogénicos en donde cada miembro ha suavizado sus métodos defensivos. Entonces esperariamos que los mecanismos de colonización y establecimiento de las bacterias endófitas se asemejaran particularmente a los mecanismos utilizados por las bacterias patógenas.

Esta tesis presenta datos novedosos sobre la regulación de la colonización causada por fertilizantes nitrogenados, la hipótesis última planteada se podría evaluar y extender en este contexto en los modelos en que las infecciones por patógenos son afectadas por niveles de nitrógeno de las plantas.

El análisis molecular de nuevos aislados, el proponer nuevas especies y el definir oligonucleótidos específicos para las mismas, contribuye al conocimiento de bacterias fijadoras de nitrógeno en la familia Acetobacteraceae.

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ANEXO I.

Artículo "Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization".

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Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization

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Abstract

Acetobacter diazotrophicus is a nitrogen-fixing endophytic bacterium, originally isolated from sugarcane. Its colonizing ability was evaluated in high and low N-fertilized sugarcane plants by inoculating stem-cuts with a β-glucuronidase marked *A. diazotrophicus* strain. Bacterial quantification by the most probable number technique showed a severe decrease of *A. diazotrophicus* cells in plants fertilized with high levels of nitrogen. The inoculated strain was detected inside low N-fertilized sugarcane plants by histochemical staining of β-glucuronidase and scanning electron microscopy. *A. diazotrophicus* was found mainly inside cortical cells of stems and inside xylem vessels. No β-glucuronidase activity was observed in non-inoculated plants. High nitrogen fertilization of fields might be a threat to maintaining naturally occurring endophytic associations.

Keywords: *Acetobacter*; Auxin; Endophyte; Nitrogen-fixing bacteria; Sugarcane; Xylem

1. Introduction

The sugarcane crop is vegetatively propagated by use of stems and this plant produces large amounts of biomass which demand a massive input of nutrients, especially N and K [1]. In almost all countries where this crop is cultivated, a common agricultural practice is to apply 250 kg N or more per Ha. Nevertheless, Brazilian farmers have used amounts of fertilizer that do not adequately cover the theoretical loss of nitrogen occurring when the plants are harvested. Surprisingly, these crops do not show nitrogen deficiencies, and their response to the addition of nitrogen fertilizer is usually negligible [2]. Consequently, biological nitrogen fixation (BNF) has been suggested to contribute to the nutrition of sugarcane plants [3]. In fact, experiments using ^{15}N isotope dilution or N balance methods gave evidence that BNF provided an important proportion of the nitrogen requirements of different sugarcane varieties [4,5].

Different N_2-fixing bacteria, such as *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella pneumoniae*,

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Azotobacter vinelandii, *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*), *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and *Acetobacter diazotrophicus* colonize the sugarcane rhizosphere and inner tissues [3,6,7]. These bacteria, and possibly other diazotrophs not yet isolated could contribute to BNF in this plant. It appears that when the stalks are sown, they carry endophytic bacteria that may spread inside the plant after budding. *A. diazotrophicus* has been suggested to be an endophytic contributor of nitrogen to this crop, as it fixes nitrogen in culture medium under acidity levels and sugar concentrations that resemble those inside the plant [6,8,9]. It has been reported that the frequency of isolation of *A. diazotrophicus* from sugarcane plants diminishes in relation to the amounts of N-fertilization used in the fields [10,11]. Caballero-Mellado et al. [12] found that Brazilian isolates were genetically more diverse than Mexican ones and suggested that this could be related to the difference in nitrogen fertilization levels between the two countries, in such a manner that the application of more fertilizer caused a diminished diversity. In the nitrogen-fixing symbiosis of *Rhizobium* and legumes, high nitrogen fertilization abolishes nodulation or, when applied to existing nodules, nitrogen fixation. It was therefore of interest to evaluate if the supposedly nitrogen-fixing *A. diazotrophicus*-sugarcane association was similarly affected by nitrogen.

By using microscopic techniques in root-inoculated sterile plantlets, James et al. [13] detected *A. diazotrophicus* colonizing the root intercellular spaces, and the interior of root epidermal cells. They proposed that *A. diazotrophicus* could be distributed from the base of the stem to other organs via the stem xylem vessels, since they also detected xylem colonization in the basal region of the stalk. In non-inoculated sugarcane plants, Dong et al. [14] isolated *A. diazotrophicus* from apoplastic fluid, that includes fluid from various locations, such as cell walls, intercellular spaces, and xylem sap [15].

The aim of this work was to examine the effects of nitrogen fertilization on the endophytic colonization of the sugarcane by inoculating an *A. diazotrophicus gusA* marked strain. In addition, we attempted to clarify the location of *A. diazotrophicus* inside the plant.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains were cultured in Luria–Bertani medium at 37°C. When necessary kanamycin (Km), streptomycin (Sm), spectinomycin (Sp), and nalidixic acid

Table 1
Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
CMK	Nal^s, Sm^s	[41]
A. diazotrophicus		
UAP 5541	Wild-type, without plasmids, able to fix N_2 in vitro, common clone by multilocus assay, Nal^r	[10,18]
Plasmids		
pRK2013	Km^r, Tra^+, ColE1 replicon, helper plasmid	[42]
pRG960SD	Sm^r, Sp^r cosmid, IncP, Mob^+, promoter-less *gusA* with a Shine and Dalgarno sequence	[17]
pBI426	Ap^r, Km^r vector with *gusA*-NPTII expressed from a double 35S CaMV virus promoter plus a leader sequence from alfalfa mosaic virus, also *gusA*-NPTII expressed from unidentified region in different Gram-negative bacteria	[16]
pRGS561	Sm^r,Sp^r, pRG960SD derivative with *gusA*-NPTII constitutive expression in *A. diazotrophicus*	This work

[a]Nal, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Km, kanamycin; Ap, ampicillin; s, sensitive; r, resistant.

(Nal) were added at final concentrations of 25, 60, 60, and 15 μg ml^{-1}, respectively. LGI medium [6] was used for growing *A. diazotrophicus* strains, and N-free semi-solid LGI [6] for the isolation of the inoculated strains. For triparental conjugations, MESMA medium with the following composition was used (g l^{-1}): yeast extract, 2.7; glucose, 2.7; mannitol, 1.8; MES (Sigma, St. Louis, MO), 4.4; K_2HPO_4, 4.81; KH_2PO_4, 0.65; Bromothymol blue, 0.025; and agar, 12; pH 6.7. For measuring the GUS activity of *A. diazotrophicus*, cells were grown in LGI broth, containing (g l^{-1}): K_2HPO_4, 0.2; KH_2PO_4, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeCl_3 \cdot 6H_2O$, 0.01; $Na_2Mo_4 \cdot 2H_2O$, 0.002; and $(NH_4)_2SO_4$, 0.617; plus the carbon source previously filter-sterilized (sucrose, fructose, glucose or gluconate, 1.5%), pH 5.5. GUS activity was also determined in cells growing with sucrose (10%) added to semi-solid SUCMES, containing (g l^{-1}): MES 4.4, K_2HPO_4 4.81, KH_2PO_4 0.65, $(NH_4)_2SO_4$ 1.48, and agar 1.5, pH 6.7.

2.2. Plasmids and strain construction

To construct a *gusA* marked strain, a DNA fragment from pBI426 [16] carrying an alfalfa mosaic virus leader sequence and a double 35S CaMV promoter fused to *gusA*-NPTII, was inserted into the broad-host range plasmid pRG960SD digested with *Eco*RI–*Hin*dIII [17]. The resulting construct (pRGS561) was conjugatively mobilized from *E. coli* to *A. diazotrophicus* strain UAP 5541 [10,18] by triparental mating using *E. coli* HB 101/pRK2013 as a helper. *A. diazotrophicus* transconjugants were selected on MESMA plates containing Nal (15 μg ml^{-1}) and Sm (45 μg ml^{-1}).

2.3. Fluorogenic β-glucuronidase assays

The β-glucuronidase (GUS) activity of *A. diazotrophicus* UAP 5541 carrying pRGS561 was tested in vitro with a fluorogenic assay as described by Jefferson [19]. The wild-type strain *A. diazotrophicus* UAP 5541 and its derivative were grown with different carbon sources at 1.5% concentration (sucrose at 1.5 and 10%). Bacterial cells were resuspended in extraction buffer containing 50 mM sodium phosphate, pH 7.0; 10 mM β-mercaptoethanol; 10 Mm Na_2EDTA; 0.1% *N*-lauroylsarcosine; and 0.1% Triton X-100. Bacterial extracts were incubated at 37°C in MUG buffer, consisting of 1 mM 4-methylumbelliferyl-D-glucuronide (Sigma, St. Louis, MO) in extraction buffer. Aliquots were removed every 5 min for 30 min. The reaction was stopped by mixing aliquots with 0.2 M Na_2CO_3. For each assay, a calibration curve was preformed with 100 nM 4-methylumbelliferone (MU) in extraction buffer. Fluorescence determinations were performed with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA), at wavelengths of 365 nm (excitation) and 460 nm (emission).

2.4. Inoculation and growth of plants

Adult stalks of the sugarcane varieties Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418, regenerated from tissue cultures and subsequently grown in experimental fields, were kindly supplied by R. Méndez-Salas (Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Zacatepec, Mexico). This plant material was selected for the colonization experiments as it was shown to lack endogenous β-glucuronidase activity.

A. diazotrophicus strains were introduced inside sugarcane stems (setts) prior to their budding so as to resemble the *A. diazotrophicus*–sugarcane relationship under natural conditions. Setts having one node were inoculated with bacteria suspended in water. The setts were previously dehydrated at 45°C for 8–10 h. Approximately 10^7 bacterial cells were inoculated per plant. The setts were planted in sterile humid vermiculite/perlite mixture (1:1), incubated in a greenhouse at 28°C, and watered with sterile water until they began to bud. After budding, 50 ml of MS modified mineral solution [20] was added weekly per plant, for ten times maximum. The mineral solution contained the following: 1.5 mM $MgSO_4 \cdot 7H_2O$, 100 μM H_3BO_3, 30 μM $ZnSO_4 \cdot 7H_2O$, 100 pM $CuSO_4 \cdot 5H_2O$, 5.3 μM KI, 105 pM $CoCl_2 \cdot 6H_2O$, 1 μM $Na_2MoO_4 \cdot 2H_2O$, 100 mM $MnSO_4 \cdot H_2O$, 3 mM $CaCl_2 \cdot 2H_2O$, 4.1 mM Na_2EDTA, 6.7 mM $FeSO_4 \cdot 7H_2O$, and 1.3 mM potassium phosphate, pH 6.0. Nitrogen fertilizer was supplied every 2 weeks, with the high and low treatments consisting of 11 and 0.56 mmol of NH_4NO_3 per plant, respectively. The plants used for histo-

chemical GUS assays, inoculated strain isolation and scanning electron microscopy were collected 1, 2, 3, 5 or 7 months after sprouting.

2.5. Histochemical β-glucuronidase analysis

The histochemical assay was done as recommended by Jefferson and Wilson [21]. Each plant sample from all the varieties tested was aseptically separated into two subsamples. Sections of stem and roots from one subsample were incubated in the following buffer: 2 mM X-Gluc (Biosynth, Staad, Switzerland), previously dissolved in DMSO, 100 mM sodium phosphate pH 7.0, 0.5 mM Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 10 mM Na_2EDTA, and 2 nM $Na_2S_2O_5$. Sections from the other subsample were incubated in the same buffer supplemented with 0.02% NaN_3. The sections were incubated for 24 h at 30°C and used for microscopical analysis.

2.6. Scanning electron microscopy

Plant samples were fixed in 3% glutaraldehyde in 100 mM sodium phosphate pH 7.0, washed with 100 mM sodium phosphate pH 7.0, fixed in 1% osmium tetroxide, washed again with phosphate buffer and predehydrated with increasingly concentrated ethanol (from 30 to 99%). The specimens were dehydrated to critical point and gold coated. Observations were carried out in a JSM-5410LV (Jeol, Tokyo, Japan) scanning electron microscope.

2.7. Optical microscopy

At each sampling time (10 days, 1, 2, 3, 5 and 7 months after sprouting) stems and roots of two plants assayed for GUS activity were observed under low magnification. Two sections from each stem of plants without GUS activity and two stem sections with GUS activity were selected for examination at higher magnifications. Samples were fixed in glutaraldehyde, washed in 100 mM sodium phosphate pH 7.0 and predehydrated with ethanol as described above. Samples were immersed in propylene oxide, and in 1:1 propylene oxide-Eponate 12 resin (Pelco, Redding, CA) mixture, and embedded in Eponate 12. Polymerization was carried out overnight at 60°C and 1.5 μm sections were used for observation. From the fixed samples three subsections were taken from two different non-inoculated and two inoculated plants which had been grown under two nitrogen fertilizer doses.

2.8. A. diazotrophicus re-isolation

A. diazotrophicus was isolated from inoculated sugarcane plants as described previously [10]. Small pieces from the plant samples to be assayed histochemically for GUS activity were aseptically separated and crushed for inoculation in LGI semi-solid media. After 6 days at 30°C the bacterial growth was streaked on LGI plates and incubated at the same temperature for 5 days.

2.9. Quantification of A. diazotrophicus cells

From sugarcane plantlets cv. Z MEX 5532 (1 and 2 months after spouting), *A. diazotrophicus* cells were quantified by the most probable number method (MPN). Root and stem samples obtained from surface sterilized sugarcane plants were finely macerated and resuspended in a chilled sucrose solution (1%). Serial dilutions were inoculated by triplicate in LGI semi-solid media containing cycloheximide (150 μg ml^{-1}) and incubated at 30°C for 5–6 days. Diazotrophs were enriched by incubating under similar conditions in the same media. Positive growth of *A. diazotrophicus* was determined by acidification and formation of the typical pellicle [6]. Numbers of bacteria were normalized to fresh weight of tissue. The presence of *A. diazotrophicus* was verified by morphology in LGI plates. In addition, Sm resistance in MESMA plates and GUS activity were confirmed in isolates from plants inoculated with UAP 5541 carrying pRGS561. The plasmid was purified from 15 colonies and observed by ethidium bromide staining.

2.10. Experimental design

Two different experiments were performed to evaluate the effect of the nitrogen on *A. diazotrophicus* colonization. For the first experiment, plants (cv. Z MEX 5532) were used 30 days after sprouting. The plants for the first experiment consisted of 20 canes

grown with the low nitrogen dose and 24 canes with the high nitrogen dose. The difference between the number of *A. diazotrophicus* in canes grown under low and high nitrogen fertilization 30 days after sprouting were tested with Student's *t*-test. For the second experiment, plants 60 days after sprouting (cv. Z MEX 5532) were used. For this determination, four inoculated plants grown with the low nitrogen fertilization and four grown with the high nitrogen fertilization were processed, as well as eight non-inoculated controls, four of which were grown under the high and four under the low fertilization conditions.

Additionally, colonization was detected by re-isolating *A. diazotrophicus* from inoculated plants 1, 2, 3, 5 and 7 months after sprouting. The varieties used in this experiment were Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418.

3. Results

3.1. Colonization of sugarcane by A. diazotrophicus

The inoculated strains could be recovered and identified from low N-fertilized sugarcane varieties Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418 at 1, 2, 3, 5 and 7 months after sprouting. Isolated bacteria produced the typical *A. diazotrophicus* growth in semi-solid LGI and showed the expected colony morphology in LGI plates. The numbers of *A. diazotrophicus* that endophytically colonized sugarcane stems or roots (cv. Z MEX 5532) fertilized with different nitrogen quantities showed significant differences (Table 2). Differences in *A. diazotrophicus* colonization was also seen in 60-day plants. The numbers in low N-fertilized plants were 2.9×10^3 and 1.2×10^3 colony forming units (CFU) per g of fresh weight in the stem and in the roots, respectively, while in the high N-fertilized plants, *A. diazotrophicus* was not detected by the most probable number method. Even in older plants (up to 7 months) of five different cultivars (Z MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418), *A. diazotrophicus* could not be isolated from inoculated plants maintained under high nitrogen fertilization, while isolation was always successful from plants maintained under low nitrogen fertilization (results not shown).

No *A. diazotrophicus* isolates were obtained from non-inoculated plants. An estimate of the total number of bacteria endophytically colonizing the roots and stems of sugarcane was in a range from 4×10^6 to 40×10^6 CFU per g of plant fresh tissue. These values were obtained from the bacterial growth in the semi-solid selective medium LGI. Possibly a great proportion of these bacteria were nitrogen-fixers, since the nitrogen in semi-solid LGI medium comes only from impurities in the components. Different types of bacteria were isolated in LGI media from non-inoculated plants. None of them corresponded morphologically to *A. diazotrophicus* in semi-solid LGI or LGI plates and we did not try to define their taxonomic status.

3.2. Localization of A. diazotrophicus in planta

To follow plant colonization by *A. diazotrophicus*, a GusA$^+$ strain was obtained by using a DNA fragment with the alfalfa mosaic virus leader and the double CaMV promoter fused to *gusA* [16]. The strain showed GUS activity in vitro when grown with carbon sources, such as gluconic acid, fructose, glucose and sucrose (results not shown).

GUS activity was detected in stems, but only in plants with low nitrogen fertilization (varieties: Z

Table 2
A. diazotrophicus cell numbers colonizing inoculated sugarcane plants at 30 days after sprouting[a]

	Stems	Roots
Low N[b]	$5.7\times10^{2\,A}$ (1.75×10^2)	$2.7\times10^{2\,A}$ (1.28×10^2)
High N[c]	$0.5\times10^{2\,B}$ (0.4×10^2)	$<0.3\times10^{2\,B}$

Superscript A and B mean that difference in bacterial numbers is significant ($P>0.05$).
[a]CFU per g of fresh weight determined by most probable number counting technique.
[b]Mean of 20 plants (S.E.M. in parentheses).
[c]Mean of 24 plants (S.E.M. in parentheses).

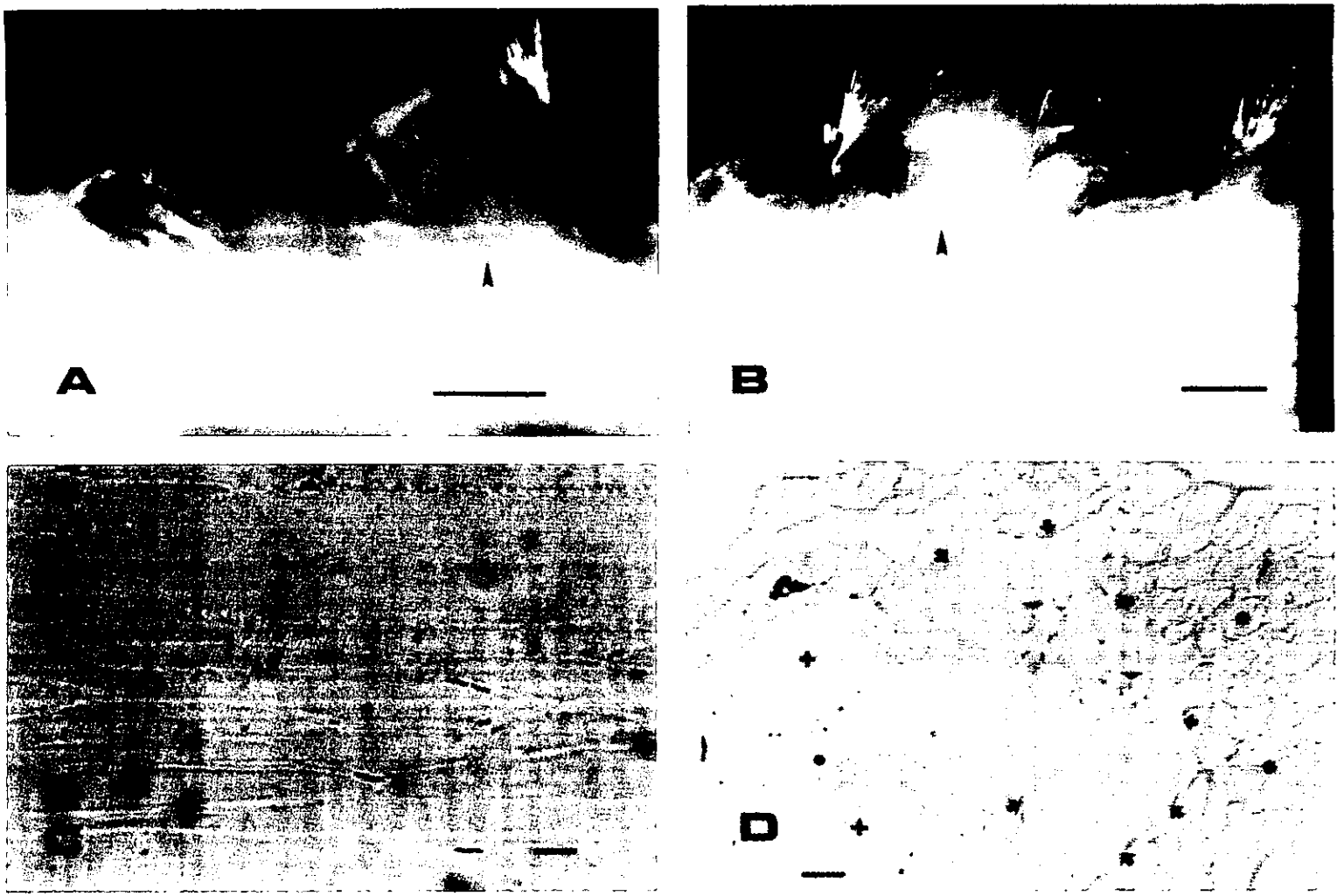

Fig. 1. GUS expression in sugarcane plants inoculated with a β-glucuronidase marked *A. diazotrophicus* strain, the GUS activity was detected with X-Gluc. Sections of stems of 2-month-old plants inoculated with the strain UAP 5541, carrying the plasmid pRGS561. (A,C,D) sections of stems of plants grown with low N-fertilization, and (B) section of a stem of a plant grown with high N-fertilization. (A,B) Low magnifications of longitudinal sections of inoculated sugarcane stems, developing buds are shown with arrows; (A) section of a stem showing GUS activity; (B) section of a stem with no GUS activity. (C) Longitudinal section of a vascular bundle showing a xylem vessel (*), the secondary wall growth of the vessel is shown with arrows. (D) Transversal section of a vascular bundle, two metaxylem vessels (+) a protoxylem (○) the sclerenchymatous sheath (*) and the phloem, delimited with arrows, are shown; the principal GUS activity is observed in phloem sieve tubes (▼). Scale bars: A and B, 0.5 mm; C and D, 20 μm.

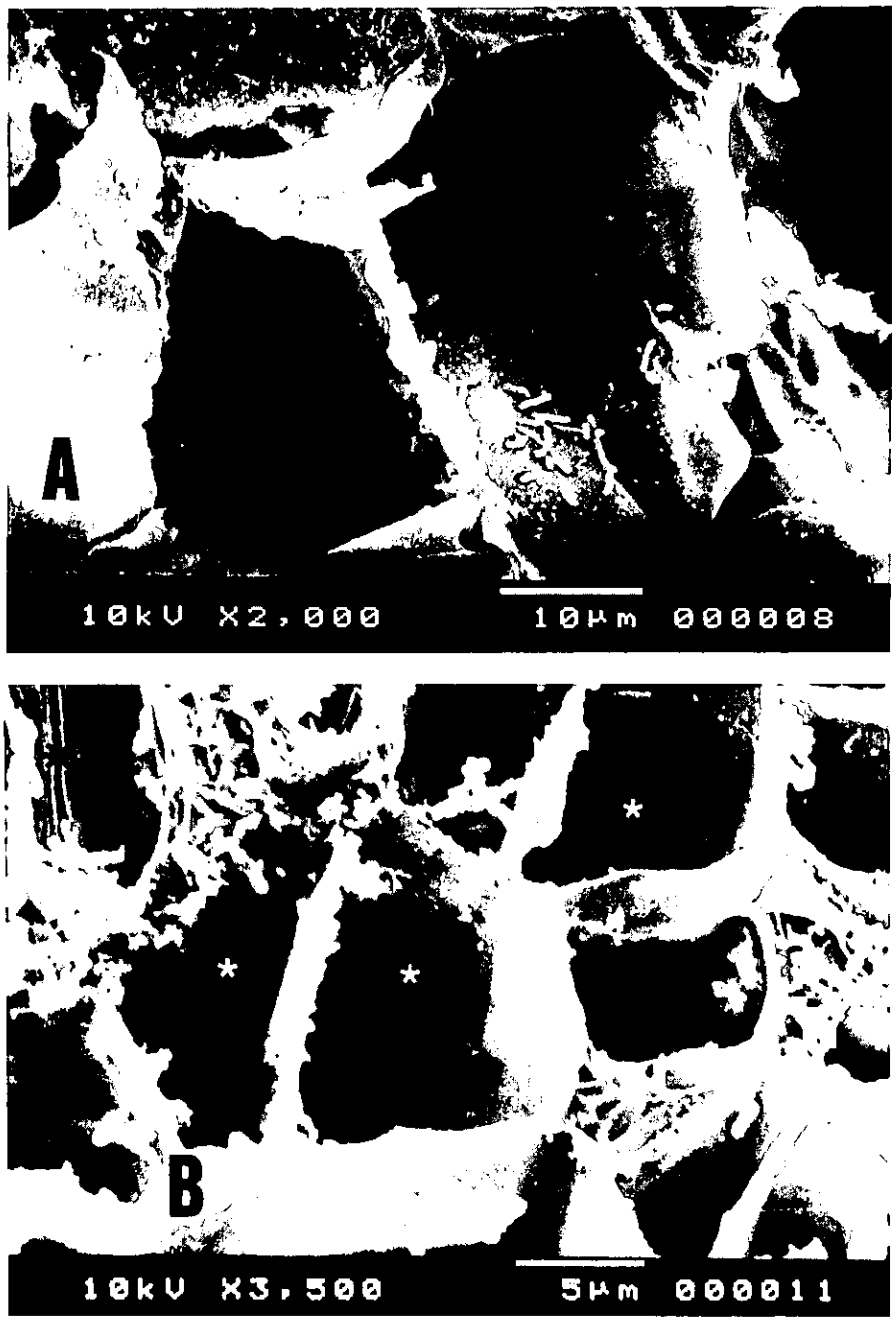

Fig. 2. *A. diazotrophicus*-like cells inhabiting cells of sugarcane stems. Sections of 5-month-old plants grown with low N-fertilization. (A) Section of sucrose storage parenchymatous cells located near to the stem cortex. (B) Section of a vascular bundle, a tracheary element (black asterisk) is surrounded by parenchyma cells (white asterisks).

MEX 5532, MEX 57-473, RD 75-11, MY 55-14, and RB 76-5418). Only in assays without NaN_3 was GUS activity detected. In stem tissues, the highest GUS activity was present in the cortex and the vascular bundle (xylem vessels and apparently also phloem sieve tubes), (Fig. 1A,C,D). GUS activity was never observed in non-inoculated plants (not shown). Scanning microscopy of stem samples from plants inoculated with the strain UAP 5541 carrying pRGS561 showed bacterial cells adhering to the walls of plant cells (Fig. 2). These bacteria were morphologically indistinguishable from typical *A. diazotrophicus* rods (0.6×2 μm). The vascular bundle and its surrounding cells were most abundantly colonized by these cells. In the non-inoculated controls, it was also possible to observe bacterial cells (not shown), but they were clearly different in size and shape from the *A. diazotrophicus* cells.

4. Discussion

The internal colonization of sugarcane by *A. diazotrophicus* in plants maintained with low and high nitrogen fertilizer doses was evaluated with a *gusA* Sm^r strain by re-isolation of the strain, confirmation of its identity by histochemical staining for β-glucuronidase and antibiotic resistance, and by scanning electron microscopy.

We used plants originally regenerated from tissue culture because they lacked endogenous GUS activity. GUS activity was obtained from stems of different sugarcane varieties coming from agricultural fields, and it is most probably of bacterial origin, as described in *Dioscorea* [22]. Successful colonization was observed in all the sugarcane varieties used, but the inoculated strain and the GUS activity were only detected in plants grown under low levels of nitrogen fertilization. The lack of *A. diazotrophicus* colonization in the presence of high supplied nitrogen explains the low presence and low frequency of isolation of *A. diazotrophicus* from sugarcane plants grown in fields with high nitrogen fertilization levels, reported previously by Fuentes Ramirez et al. [10] and Muthukumarasamy [11]. In experiments with the grasses *Miscanthus sinensis*, *M. sacchariflorus* and *Spartina pectinata*, Kirchhof et al. [23] found similar results by quantifying the cell numbers of the diazotrophic endophytic community inhabiting plants fertilized and unfertilized with nitrogen. This effect of nitrogen on colonization might not be universal since, for instance, *Herbaspirillum rubrisubalbicans* behaves as a pathogen in susceptible sugarcane cultivars grown in countries where high levels of nitrogen fertilization are used [7]. We detected 10^2–10^3 CFU per g of fresh tissue of *A. diazotrophicus* endophytically colonizing low N-fertilized 30-day-old sugarcane plants. In roots of non-inoculated sugarcane plants, Reis et al. [24] found 10^1–10^6 CFU of *A. diazotrophicus* per g of fresh tissue. The quantitative difference found between that report and ours could be related to the inoculation process that we used. In addition, the estimate of Reis et al. [24] could also include superficially adhering cells in addition to endophytic ones, and the plant cultivar might also have an influence [15]. In a study of the association of *A. diazotrophicus* with different cultivars of sugarcane, da Silva et al. [25] suggested that the *A. diazotrophicus* population is sensitive to the plant genotype. They observed that only in one of their cultivars the *A. diazotrophicus* population increased during the time of the study (15 months), but in the other ones, they did not detected any trend in the bacterial numbers.

The effect observed on the *A. diazotrophicus* population colonizing sugarcane does not seem to be a direct negative effect of the fertilizer on the bacteria. We did not detect negative effects on wild-type and $GusA^+$ *A. diazotrophicus* strains growing in culture media supplied with high nitrogen levels (10 mM NO_3^-), and at the same nitrogen concentration the $GusA^+$ construct also expressed β-glucuronidase activity. Thus, it is more probable that the physiological state of the plant is altered by the nitrogen, and this subsequently affects its association with the endophyte. Pelaez Abellan et al. [26] observed that sucrose synthesis is reduced in sugarcane leaves by application of NO_3^- in a highly productive variety and increased sucrose synthesis in a variety with low productivity. In complete sugarcane plants, high nitrate doses were associated with a decrease in the concentrations of sucrose in the leaves and of the reducing sugars and sucrose in the stem [27].

A. diazotrophicus is commonly found in roughly the same numbers in sugarcane roots, stems and leaves under field conditions [6,28,29]. In the present

study, the inoculated strains were recovered from stem and root samples, and we also detected GUS activity in the stems, but not in roots, in spite of the similar bacterial numbers recovered from both organs. We cannot explain the lack of activity in root tissue, especially considering the similar numbers of *A. diazotrophicus* cells inhabiting this organ relative to the numbers inside the stem.

In this study, the expression of GUS activity was only used as a qualitative reporter of the location of the inoculated strain. Nevertheless, the lack of GUS activity in NaN_3 treated samples suggests that the *A. diazotrophicus* population in the sugarcane was lower than the limit of detection of the assay that we previously determined in vitro (2×10^5 CFU per cm^3 of tissue). That population-size indicator supported the data that was obtained by MPN technique. This estimation does not consider that X-Gluc diffusion could be limited by the plant cell walls. Another possibility is that a low proportion of bacterial cells retain the plasmid, but this does not seem to be the case, since we found plasmid maintenance to be higher than 95% after 7 months inside the plant (not shown). The absence of a bacterial growth inhibitor (NaN_3) in the histochemical incubations probably allowed an increase in the *A. diazotrophicus* population and the detection of GUS activity. Low population densities of this bacterium in sugarcane are expected as there is no evidence of specialized plant structures which harbor high concentrations of bacteria ([13–15], and this work). It is probable that *A. diazotrophicus* is almost equally distributed inside several structures throughout the bulk of the plant, and that population growth is limited, for some unknown reason, in the sucrose-rich tissues. Higher GUS activity was observed in stem xylem vessels, phloem sieve tubes and cortex, and by scanning microscopy *A. diazotrophicus*-like rods were found in cells of the stem cortex, adhering to the inner cell wall. We presumed those cells to be *A. diazotrophicus* because of their physical similarity to cells grown in culture, and since they were found only in *A. diazotrophicus* inoculated plants and were more abundant in GUS positive sections. We do not know if the plant cells seemingly colonized by *A. diazotrophicus* were damaged or alive. By immunogold labeling, James et al. [13] detected *A. diazotrophicus* inside cells from the cortex of sugarcane plantlet roots, and inside the xylem vessels from the base of the stem. They suggested that the root xylem could be the route for stem and leaf xylem infection. Our work supports previous results from another group [13,15] in that the cavities formed by the xylem secondary wall are one of the preferentially colonized microhabitats. Dong et al. [14] have proposed that *A. diazotrophicus* is found in the intercellular spaces of the stem storage parenchyma, where there are plentiful nutrients [30]. James et al. [13] also suggested that the stem xylem and the leaf xylem might be the final colonized environments inside the cane. In addition, we present evidence that *A. diazotrophicus* could colonize the stalk cortical tissue as well as its xylem. From the xylem, the bacterium might preferentially migrate to the cells of selected tissues, such as the cortex. This ultimate distribution may provide a more favorable environment for the endophyte and its N_2-fixing activity, considering that the xylem apoplastic fluid is almost devoid of carbon sources [31]. Some pathogenic and mildly pathogenic bacteria of sugarcane also colonize and survive in the xylem elements, and from there translocate to other places [32,33]. In another work, Dong et al. [34] claimed that the xylem vessels were an improbable colonization site for *A. diazotrophicus*, since after introducing this bacterium inside the stem, the plant reacted by producing substances that may have clogged the vessels. Nevertheless, their experiment probably did not reflect the natural association between *A. diazotrophicus* and sugarcane, since they made their observations on plants that were recently stressed by wounds. Moreover, as they inoculated stems by submerging their cut ends for several days in a growing bacterial suspension, with the consequent release of metabolic products, the defense reaction observed might have been expected with any bacteria.

Dong et al. [34] asserted that the xylem vessels of sugarcane were discontinuous, preventing the transport of *A. diazotrophicus* through the xylem. We presume that even if the xylem vessels are limited in their ability to translocate particulate material, *A. diazotrophicus*, and probably other species adapted to this environment, could induce plant morphological changes, such as formation of continuous vessels, by releasing plant growth regulators. It has been previously shown that *A. diazotrophicus* produces

auxins in a minimal culture medium [10]. The hypothesis of the role of *A. diazotrophicus*, is based on the observation that during the course of xylem element formation, the walls that separate adjacent vessel cells are hydrolyzed in a process that seems to be controlled by the presence of auxins [35].

The promoter used for expression of *gusA* is known to be active in eukaryotic tissues. Nevertheless, a DNA fragment that includes a duplicated CaMV promoter plus a leader sequence of AMV (alfalfa mosaic virus) was shown to induce high β-glucuronidase activity under different conditions in *A. diazotrophicus*. The bacterial recognition of eukaryotic promoter sequences might not be entirely surprising as it is known that some plant plastid promoters share consensus sequences with −35 −10 bacterial promoters [36]. Moreover, at least one eukaryotic transcription factor (TFIID) is known to show high similarity with bacterial σ-factors [37]. Particularly, the most similar region between TFIID and the σ-factors has been suggested to interact with DNA, binding to the eukaryotic TATA box in TFIID, or to single stranded DNA and to −10 bacterial promoters, in the bacterial factors. In addition, the presence of a leader sequence could enhance the translation of the *gusA* transcript, as has been observed with mRNA in different Gram-negative bacteria [38].

Under the conditions used here, sugarcane plants up to 8 month of age showed no differences in development when inoculated with *A. diazotrophicus*. From preliminary results in our laboratory, no nitrogenase expression was detected in planta from an *A. diazotrophicus* strain containing a *nifH-gusA* fusion, nevertheless we do not discard the possibility of beneficial effects of the bacteria in plants grown under other conditions, as have been reported by Sevilla et al. [39]. *Azoarcus* sp., another endophytic diazotroph has also been located inside root cortical cells of Kallar grass and inoculated rice [40]. In this association, the authors found some beneficial effect on biomass and protein content in rice plants inoculated with this bacterium.

Endophytic relationships are becoming an interesting field for studying plant-bacteria interactions and their study is still at an initial phase. Two threats to the naturally occurring endophytic associations are the high N-fertilization levels used in the modern agriculture, and the now common use of tissue culture to propagate pathogen-free sugarcane. Both practices will probably eliminate diazotrophic bacteria, as reported in this work, and sugarcane producers should be aware of this situation.

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ANEXO II.

Artículo "Genetic structure of *Acetobacter diazotrophicus* populations and identification of a new genetically distant group".

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Genetic Structure of *Acetobacter diazotrophicus* Populations and Identification of a New Genetically Distant Group

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A total of 55 isolates of *Acetobacter diazotrophicus* recovered from diverse sucrose-rich host plants and from mealybugs associated with sugarcane plants were characterized by the electrophoretic mobilities of 12 metabolic enzymes. We identified seven different electrophoretic types (ETs), six of which are closely related within a genetic distance of 0.195 and exhibit high DNA-DNA homology. The seventh ET was largely divergent, separated at a genetic distance of 0.53, and had only 54% DNA homology to the reference strain. Strains corresponding to ET 7 could represent a distinct nitrogen-fixing species of the genus *Acetobacter*. More genetic diversity was found in isolates from Brazil than in those from Mexico, probably due to the very different crop nitrogen fertilization levels used.

Cane sugar is produced commercially in over 70 countries around the world (31). It is an important agricultural product which is used for domestic consumption and export. More than 150 by-products may be obtained from sugarcane (33). For instance, ethanol obtained by fermentation and distillation of sugarcane juice provides fuel for 4 million motor vehicles in Brazil, and 7 million other vehicles use gasohol containing 10 to 22% ethanol (5).

Commonly, very high levels of nitrogen fertilizers (120 to 300 kg of N per ha) are used in sugarcane crops in countries such as Mexico, Venezuela, Cuba, and the United States (Hawaii). In contrast, sugarcane crops in Brazil do not receive more than 50 kg of nitrogen fertilizer (37), and neither cane yields nor soil N reserves appear to diminish after decades of culture (5). Recent experiments estimated that the contribution of biological nitrogen fixation to the sugarcane cultivars ranged from 50 to 80% of total plant nitrogen (5, 52).

Nitrogen-fixing bacterial species, such as *Enterobacter cloacae*, *Bacillus polymyxa*, *Klebsiella pneumoniae*, *Azotobacter vinelandii*, and *Azospirillum* spp., are commonly isolated from different internal or external parts of sugarcane plants (37; unpublished results). Recently, other diazotrophs (*Herbaspirillum seropedicae* [3] and *Acetobacter diazotrophicus* [11, 17]) have been isolated from inside tissues of roots and stems of sugarcane. At present, which of these bacteria are the most important in plant-associated biological nitrogen fixation remains unknown. However, *A. diazotrophicus* has been suggested as a strong candidate responsible for the N_2 fixation observed in field experiments with sugarcane (5, 51).

A. diazotrophicus has also been recovered from other sucrose-rich host plants such as sweet potato (*Ipomoea batatas*) and Cameroon grass (*Pennisetum purpureum*), which are vegetatively propagated (15), as well as from different genera of mealybugs associated with sugarcane plants (1).

Multilocus enzyme electrophoresis (MLEE) has been used extensively to measure genotypic diversity and genetic structure of natural populations of many bacterial species (43). Such studies have revealed that the levels of genetic variability differ greatly among species. For instance, *Yersinia ruckeri* organisms exhibit a genetic diversity as low as 0.014 (38), while oral streptococci show a diversity as high as 0.857 (19). Between these extremes are found very different pathogenic bacterial species of plants (14), animals (4, 30), and humans (10, 13, 29, 42), as well as soil bacterial species, including *Bacillus* spp. (9, 21), a *Bradyrhizobium* sp. (6), *Pseudomonas cepacia* (27), and *Rhizobium* spp. (16, 24, 35). Genetic diversity levels have mainly been related to effective population size (28, 45) and recent evolutionary origin of the species (12, 28), along with ecological factors (27, 45) and niche specialization (14, 23, 30, 40).

Taking into account that "the characterization and understanding of natural populations of useful bacteria may save work and money in the development of low-risk, successful biotechnology" (46), we considered it of interest to extend our previous studies on genetic diversity of *A. diazotrophicus* isolated from sugarcane (8) to include bacteria isolated from other host plants such as sweet potato and *P. purpureum* and from the mealybug *Saccharicoccus sacchari*. In this work, we report the genetic relatedness among isolates recovered mainly from Mexico and Brazil. We show evidence of a new genetically distinct group.

MATERIALS AND METHODS

Isolation. *A. diazotrophicus* strains were isolated from the inside tissues of stems or roots of sugarcane plants cultivated in Mexico, as described previously (17).

Each mealybug colony, identified as *S. sacchari*, sampled from stems of independent sugarcane plants was rinsed with 0.01% (vol/vol) Tween 40 in 10 mM $MgSO_4 \cdot 7H_2O$ until the liquid was clear. Subsequently, mealybugs were immersed in 1% chloramine T for 5 min and then washed three times in 10 mM $MgSO_4 \cdot 7H_2O$. Insects were macerated in 1.0 ml of sterile distilled water, and aliquots were inoculated into media for isolation of *A. diazotrophicus*, as described previously (17).

Mealybug colonies and sugarcane varieties sampled in Mexico were from diverse cane-growing areas up to 1,500 km apart; cane-growing areas of Brazil were located up to 2,500 km apart.

Bacterial strains. Strains and their sources are shown in Table 1. Most of the strains were recovered from hosts collected in Mexico and Brazil, but the samples also included two isolates from Australia and one from Uruguay. Strain 7.10RM was recovered from within spores of the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* obtained from sweet potatoes grown in soil inoculated with a mixture of the fungus and strain PAI 5^T of *A. diazotrophicus*. Only isolates

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TABLE 1. ETs, host species, and locality for 55 isolates of *A. diazotrophicus*

ET	Strain	Host species	Locality	Source
1	CFNE 501	Stem, Z Mex 55 32[a]	Veracruz, Mexico	This work
1	CFNE 502	Stem, Mex 69 290[a]	Veracruz, Mexico	This work
1	CFNE 503	Stem, RD 75 01[b]	Veracruz, Mexico	This work
1	CFNE 504	Stem, Mex 73 523[a]	Veracruz, Mexico	This work
1	CFNE 505	Stem, Mex 68 P23[a]	Sinaloa, Mexico	This work
1	CFNE 506	Stem, RD 75 11[b]	Sinaloa, Mexico	This work
1	CFNE 507	Stem, RB 73 9953[b]	Sinaloa, Mexico	This work
1	CFNE 508	Stem, SP 70 1005[b]	Sinaloa, Mexico	This work
1	CFNE 509	Stem, SP 70 3370[b]	Sinaloa, Mexico	This work
1	CFNE 510	Stem, RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 513	Stem, My 55 14[b]	Puebla, Mexico	This work
1	CFNE 515	Stem, SP 70 1248[b]	Puebla, Mexico	This work
1	CFNE 516	Roots, SP 70 1248[b]	Puebla, Mexico	This work
1	CFNE 521	Roots, CP 72 2086[b]	Veracruz, Mexico	This work
5	PAI 3	Roots, sugarcane	Alagoas, Brazil	CNPAB collection[c]
1	PRJ 6	Roots, CB 47 89	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 14	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 17	Stem, IAC 52 150	Rio de Janeiro, Brazil	CNPAB collection
3	PRJ 20	Stem, Na 56 79	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 24	Roots, RB 73 9735	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 36	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 40	Leaves (trash), CB 36 14	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 54	Stem, Krakatau	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 56	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
3	PRX 3	Xylem, CB 45 3	Rio de Janeiro, Brazil	CNPAB collection
3	PRX 6	Xylem, Na 56 79	Rio de Janeiro, Brazil	CNPAB collection
3	PSP 15	Roots, Na 56 79	São Paulo, Brazil	CNPAB collection
4	PSP 22	Leaf, Na 56 79	São Paulo, Brazil	CNPAB collection
3	PSP 32	Stem, Na 56 79	São Paulo, Brazil	CNPAB collection
6	PSP 17	Rhizoplane, Na 56 79	São Paulo, Brazil	CNPAB collection
3	PSP 19	Rhizoplane, Na 56 79	São Paulo, Brazil	CNPAB collection
1	URU	Roots, sugarcane	Uruguay	CNPAB collection
7	LMG 1733	Sugarcane	Australia	CNPAB collection
1	CFNE 530	Mealybugs-PT 49 143[b]	Veracruz, Mexico	This work
1	CFNE 531	Mealybugs-PT 49 143[b]	Veracruz, Mexico	This work
1	CFNE 532	Mealybugs-Z Mex 55 32[a]	Veracruz, Mexico	This work
1	CFNE 533	Mealybugs-L 78 56[b]	Veracruz, Mexico	This work
1	CFNE 534	Mealybugs-Mex 68 P23[a]	Sinaloa, Mexico	This work
1	CFNE 535	Mealybugs-RD 75 11[b]	Sinaloa, Mexico	This work
1	CFNE 537	Mealybugs-RB 73 9953[b]	Sinaloa, Mexico	This work
1	CFNE 539	Mealybugs-RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 541	Mealybugs-RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 542	Mealybugs-RB 72 1022[b]	Sinaloa, Mexico	This work
1	CFNE 544	Mealybugs-RB 73 9953[b]	Sinaloa, Mexico	This work
2	CFNE 550	Mealybugs-CB 45 3[d]	Rio de Janeiro, Brazil	This work
2	CFNE 554	Mealybugs-CB 45 3[d]	Rio de Janeiro, Brazil	This work
6	1772	Mealybugs	Ayr, Australia	M. W. Dawson[e]
1	PBD 4	Tuber, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 13	Peel, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 16	Roots, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 17	Tuber, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
3	Pcol	Stem, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
7	PRC 1	Stem, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
7	PRC 4	Roots, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
3	7.10RM	Spores, VAM fungus[f]	Rio de Janeiro, Brazil	CNPAB collection

[a] Commercial sugarcane varieties.
[b] Sugarcane germoplasm.
[c] CNPAB, Centro Nacional de Pesquisa de Agrobiologia, Rio de Janeiro, Brazil.
[d] Collected from sugarcane grown in a concrete tank (5).
[e] M. W. Dawson, Sugar Research Institute, Mackay, Queensland, Australia.
[f] VAM, vesicular-arbuscular mycorrhizal.

recovered from different plants or mealybug colonies were considered to be different. Strain 1772 was kindly supplied by M. Dawson. Strains UAP 5560, PAI 5[T] (= ATCC 49037[T] [T = type strain]), and PPe 4 (= ATCC 49038) of *A. diazotrophicus*, corresponding to electrophoretic type (ET) 1, ET 3, and ET 4 as we described previously (8), were included as references in MLEE assays.

Culture media. *A. diazotrophicus* isolates and *Escherichia coli* HB 101 were grown in SYP medium (8) for all assays.

Preparation of cell extracts and MLEE. Each isolate was grown in 25 ml of SYP medium at 29°C and harvested by centrifugation, and pellets were suspended in 0.3 ml of 10 mM $MgSO_4 \cdot 7H_2O$ and treated as described previously (8).

Starch gel electrophoresis and the selective staining of 12 metabolic enzymes were done by methods described before (43). The enzymes assayed were the same ones used in a previous report (8), except for an unidentified dehydroge-

TABLE 2. Genetic diversity among isolates and ETs at 12 enzyme loci

Enzyme locus[a]	No. of alleles	Genetic diversity (H)[b] of:		No. of alleles	Genetic diversity (H) of:	
		55 isolates	7 ETs		49 isolates[c]	6 ETs[d]
IPO	1	0.000	0.000	1	0.000	0.000
LYD	2	0.197	0.285	1	0.000	0.000
LED	2	0.197	0.285	1	0.000	0.000
XDH	2	0.197	0.285	1	0.000	0.000
MDH	2	0.197	0.285	1	0.000	0.000
ADH	2	0.197	0.285	1	0.000	0.000
UDH	2	0.197	0.285	1	0.000	0.000
IDH	2	0.036	0.285	2	0.041	0.332
G6P	2	0.235	0.285	2	0.279	0.332
PGM	4	0.173	0.713	4	0.194	0.799
HEX	2	0.036	0.285	2	0.041	0.332
EST	3	0.103	0.523	3	0.119	0.600
Mean	2.16	0.147	0.316	1.66	0.056	0.199

[a] IPO, indophenol oxidase; LYD, lysine dehydrogenase; LED, leucine dehydrogenase; XDH, xanthine dehydrogenase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; UDH, unidentified dehydrogenase; IDH, isocitrate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; HEX, hexokinase; EST, esterases.

[b] $H = (1 - \Sigma x_i^2)[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of isolates or ETs.

[c] Excluding six isolates represented by ET 7.

[d] Excluding ET 7.

nase. This enzyme was visualized on gels stained for indophenol oxidase, which is revealed as white bands in the presence of light. In contrast, the unidentified dehydrogenase was observed as a typical purple band, like other dehydrogenases. We did not attempt to determine the substrate(s) for this enzyme. For all assays the electrophoretic buffer system used was Tris-citrate (pH 8.0). Distinctive combinations of alleles for the 12 enzyme loci (multilocus genotypes) were designated different ETs (43). The level of genetic diversity for each enzyme locus was calculated as described by Selander et al. (43).

Statistical analysis. The extent of linkage disequilibrium, or nonrandom association of alleles, in the studied population was evaluated to explore the degree of clonality. The ratio of the variance in mismatches observed (V_o) to the expected variance (V_e) in the population was iterated 10,000 times by a Monte Carlo procedure, as proposed by Souza et al. (47).

Total DNA isolation, DNA restriction, and filter blot hybridization. Total DNA was isolated as described previously (2). DNA was digested with *Eco*RI, and restriction fragments were electrophoresed, blotted, and hybridized as previously reported (8). DNA-DNA homology was based on relative levels of hybridization to ^{32}P-labelled DNA from *A. diazotrophicus* PAI 5^T. DNA amounts in gels and radioactivity levels were quantified as described before (26).

The restriction fragment length polymorphism patterns of the rRNA operons were determined from *Eco*RI DNA digests hybridized with a *Hin*dIII-*Hin*dIII 700-bp internal fragment from *E. coli rrnB* 16S rRNA genes cloned in pKK3535 (7).

RESULTS

MLEE and genetic diversity. A total of 55 isolates of *A. diazotrophicus* were examined, and 11 of the 12 enzyme loci analyzed were found to be polymorphic. The mean number of alleles was 2.16 (range, 1 to 4) (Table 2). A total of seven distinctive ETs were identified (Table 3). Most of the isolates (35 of 55; 64%) were identical, corresponding to ET 1. ET 4 and ET 5 were represented by only one isolate; ET 2 and ET 6 were each represented by two isolates only. Only strains corresponding to ET 1 were recovered from mealybug colonies associated with seven different sugarcane varieties and from 13 sugarcane varieties sampled in Mexico (including 5 Brazilian varieties); in contrast, seven ETs were identified from 7 sugarcane varieties cultivated in Brazil and from associated mealybugs (Table 1). Six of the ETs differed from one another at only one or two loci. However, strains grouped in ET 7 were very different from all other isolates, showing six unique alleles (Table 3). These strains were recovered from both *P. purpureum* and sugarcane sampled in Brazil, and one strain (LMG 1733) was isolated from sugarcane in Australia.

The mean level of genetic diversity per locus (H) among the seven ETs was found to be 0.316. However, the genetic diversity among isolates was lower ($H = 0.147$) (Table 2), reflecting the fact that four of the ETs were represented by one or two isolates, while only three ETs (ET 1, ET 3, and ET 7) represented 49 isolates (Table 3). Excluding ET 7, because it is largely divergent from all of the other ETs (see below), the H level among the six ETs was 0.199, and among isolates it was as low as 0.056 (Table 2), since in this case only two ETs (ET 1 and ET 3) represented 88% of the isolates.

The genetic relationships among the seven ETs are summarized by a dendrogram in Fig. 1. Six ETs (ET 1 to ET 6) were closely related, forming a cluster at a genetic distance of 0.195. A second line (ET 7), which contained six strains, was largely divergent, and it was separated by a genetic distance of 0.53.

DNA homology and ribosomal hybridization restriction fragment length polymorphisms. Six *A. diazotrophicus* strains from the closely related ETs 1 to 6 constituted a homogeneous group with relative levels of DNA homology ranging from 73 to 90% (mean homology, 86%) with reference strain PAI 5^T. This mean homology value was very similar to the level of 84% DNA homology previously determined by Gillis et al. (18) among three representative *A. diazotrophicus* strains, including the type strain PAI 5^T. The six strains corresponding to the more distant ET 7 (Table 1) exhibited only 54% homology to the same reference strain.

Strains CFNE 501, PAI 5^T, PSP 22, PAI 3, PRC 1, and LMG 1733 were analyzed by restriction fragment length polymor-

TABLE 3. Allele profiles at 12 enzyme loci in seven ETs of *A. diazotrophicus*

ET	Reference strain	No. of isolates	Allele at indicated enzyme locus[a]											
			IPO	LYD	LED	XDH	MDH	ADH	UDH	IDH	G6P	PGM	HEX	EST
1	CFNE 501	35	1	2	3	2	2	2	2	1	4	5	1	3
2	CFNE 550	2	1	2	3	2	2	2	2	1	4	6	1	3
3	PAI 5^T	8	1	2	3	2	2	2	2	1	5	5	1	3
4	PSP 22	1	1	2	3	2	2	2	2	2	4	5	2	3
5	PAI 3	1	1	2	3	2	2	2	2	1	4	7	1	1
6	1772	2	1	2	3	2	2	2	2	1	4	4	1	4
7	PRC 1	6	1	1	2	1	1	1	1	1	4	5	1	3

[a] IPO, indophenol oxidase; LYD, lysine dehydrogenase; LED, leucine dehydrogenase; XDH, xanthine dehydrogenase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; UDH, unidentified dehydrogenase; IDH, isocitrate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; HEX, hexokinase; EST, esterases.

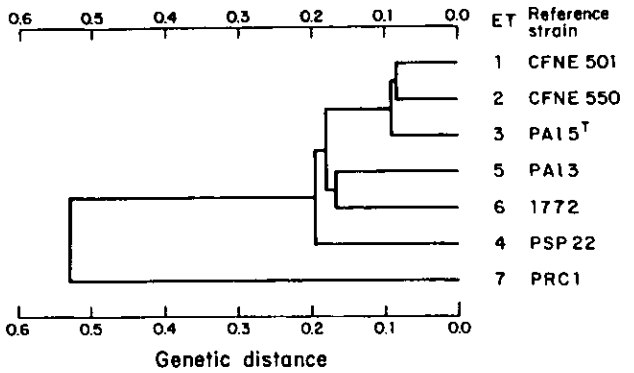

FIG. 1. Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from different hosts.

phisms of the rRNA operons. The hybridizing patterns were identical. Four common hybridizing bands (9.3, 3.6, 2.3, and 1.6 kb) were observed in all of the isolates examined (Fig. 2). Similarly, all strains showed a common pattern (8) of hybridization to *nifHDK* (data not shown).

Linkage disequilibrium. A total of 1,485 pairwise comparisons are possible among the 55 isolates. The observed variance in proportion of mismatches was 5.806, and the expected variance was 1.450. The ratio of the observed variance in numbers of mismatches to the expected variance (V_o/V_e) was 4.003, highly significant, indicating a strong linkage disequilibrium. The analyses done separately for the populations recovered from sugarcane cultivated in Brazil and Mexico revealed a strong linkage disequilibrium also (data not shown).

DISCUSSION

In this study, we report that there is a lower genetic diversity in *A. diazotrophicus* recovered from different host species collected in widely separated regions of the world in comparison to the majority of other bacterial species studied (6, 9, 10, 13, 14, 19, 29, 35, 44). The results confirm previous data (8) on genetic diversity among 21 Mexican and 3 Brazilian isolates exclusively recovered from sugarcane plants. In addition, a new genetically distant group was found.

Coefficients of genetic distance at levels higher than 0.5 have been used as a criterion to suggest species limits (27, 45). DNA-DNA hybridization levels below 60 to 70% are also indicative of separate species (39, 48). On the basis of these facts, our MLEE studies suggest that the strains represented by ET 7 could represent a distinct nitrogen-fixing bacterial species. This result was consistent with the level of DNA-DNA homology obtained. In other cases, the estimates of genetic relatedness of strains obtained by both DNA-DNA hybridization and MLEE are closely correlated (24, 32, 41, 45). Nevertheless, restriction fragment length polymorphism patterns of ribosomal genes showed that ET 7 is related to the main *A. diazotrophicus* cluster. Due to the conserved nature of the 16S rDNA sequences, the method may be limited in the differentiation of closely related species (22). Furthermore, "DNA hybridization is acknowledged as the superior method for the elucidation of relationships between closely related taxa, such as strains and species" (48). Further work will be required to define the taxonomic status of ET 7 strains.

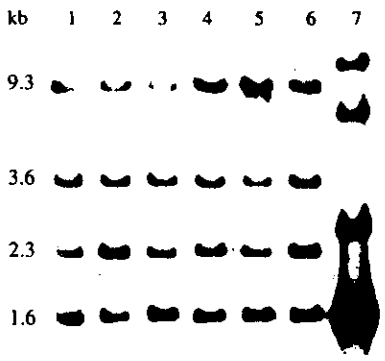

FIG. 2. Autoradiogram of a Southern blot of the total *Eco*RI DNA fingerprints hybridized with a 16S rDNA probe of *E. coli*. Lanes 1 through 6, strains CFNE 501 (ET 1), PAL 5^T (ET 3), PSP 22 (ET 4), PAL 3 (ET 5), PRC1, and LMG 1733 (ET 7), respectively; lane 7, *E. coli* HB 101, used as a control.

The restricted genetic variability observed in *A. diazotrophicus* suggests that this species has a recent evolutionary origin. Another possible explanation for the limited genetic diversity is related to the predominantly endophytic habitat of *A. diazotrophicus*, as suggested before (8), in association with niche specialization, because this species has been isolated exclusively from sucrose-rich host plants (15, 25) and from mealybugs associated with sugarcane plants (1). It has been postulated that each ecological niche acts as a selective force toward those properties of the organism that enable it to occupy that niche. Thus, the nonrandom variation suggests that the organisms are selected from those occupying closely related niches rather than very different niches (20). We do not discount that the limited genetic diversity observed in *A. diazotrophicus* could be related to the analysis of a limited subset of clones of the species, as advanced to explain the genetic diversity in other bacterial species (28, 45). This hypothesis is based on the very high selectivity of the medium used for bacterial isolation (15, 17, 36), which could influence the selection of subsets of all genotypes existing in nature. It has been observed that isolated soil bacteria make up only a very small proportion of the total bacterial community, but the largest proportion cannot be isolated or cultured on laboratory media (50).

Overrepresentation of a particular multilocus genotype is often the strongest and most significant evidence of clonality (49), particularly when the same genotype is recovered at many different localities and at different times (30, 45). The frequent recovery of isolates corresponding to the same ET from widely separated geographic regions, as well as from different hosts at different times, indicated that the genetic structure of *A. diazotrophicus* is basically clonal. This result was supported by the occurrence of a strong linkage disequilibrium in the natural population of these bacteria at both global and local levels.

The extensive distribution of closely related strains of *A. diazotrophicus* from widely separated areas of the world suggests that this bacteria was recently dispersed, as has been observed similarly in *Pseudomonas syringae* pv. tomato (14). Taking into account the endophytic characteristics of *A. diazotrophicus* and the association of the bacteria with mealybugs and vesicular-arbuscular mycorrhizal spores, we previously (8) explained the long-distance dispersal and spread among cane cultivars of this species.

It was previously suggested (1) that *A. diazotrophicus* may be autochthonous microbiota of mealybugs associated with sugarcane and other plants. However, we were able to isolate the bacteria from only 30 of 80 mealybug colonies of *S. sacchari*, including actively feeding adults, collected from stems of many different sugarcane varieties cultivated in both Brazil and Mexico. This fact suggests that *A. diazotrophicus* is sucked from sugarcane plants by the associated mealybugs, which is further

supported by our results showing that the *A. diazotrophicus* population recovered from *S. sacchari* is a subset of the *A. diazotrophicus* sugarcane population. The genetic diversity of *A. diazotrophicus* from other genera of mealybugs such as *Dysmicoccus brevipes* and a *Planococcus* sp. has not been analyzed, but perhaps it would be not surprising to find a limited genetic variability in these populations as well.

The comparison of the number of ETs identified in the collections of isolates recovered from sugarcane and mealybugs sampled in Brazil and Mexico showed that the population of *A. diazotrophicus* collected in Brazil, represented by seven ETs, is more heterogeneous genetically than the population collected in Mexico, represented only by ET 1. This apparent greater genetic heterogeneity may be related to the very different nitrogen fertilization levels that are applied to sugarcane field crops in Mexico in comparison to Brazil. A close relationship between nitrogen fertilization rates and isolation frequency of *A. diazotrophicus* was previously observed (17). At the highest fertilization rates (300 kg of N per ha), isolation frequencies of 0 to 2% were obtained, while at levels of 120 kg of N per ha frequencies increased up to 70%. Moreover, although Li and Macrae (25) did not mention any relation between isolation frequency of *A. diazotrophicus* and nitrogen fertilization, we noted that, in their results reported in Table 1, the number of isolates of this bacterium was nearly five times higher in the same sugarcane variety (CP 44101) with no N fertilizer than in N-fertilized plants collected in the same region and on the same date. Taking these observations into account, nitrogen seems to be a selective factor for certain lineages or clones of *A. diazotrophicus*. A role for a selective factor(s) may be supported in view of the endophytic nature of *A. diazotrophicus* organisms (25, 36), which supposedly are dispersed long distance inside sugarcane germoplasm commonly exchanged between countries (e.g., germoplasm of Brazilian varieties cultivated in Mexico [Table 1]). Therefore, in the absence of such a selective factor, different clones recovered in a country could be recovered from sugarcane germoplasm propagated in widely separated geographical areas. The nitrate levels available to the plant may increase or diminish the sucrose content depending on the sugarcane cultivar (34). This may explain to some extent the nitrogen fertilization effects on *A. diazotrophicus* populations, considering that sucrose is the best carbon source required in high concentration for optimal bacterial growth (11, 18). However, we could not exclude that other ecological factors, besides nitrogen fertilization rates, may contribute to the differences in genetic diversity of the *A. diazotrophicus* populations encountered in Brazil and Mexico.

Since a low number of isolates recovered from Cameroon grass and sweet potato were analyzed, it was not possible to determine if certain ETs of *A. diazotrophicus* are predominantly associated with a particular host species, as observed, for instance, with the pathogen of mammals *Bordetella bronchiseptica* (28) or with the legume-nodulating *Bradyrhizobium* sp. (6). However, the results clearly demonstrated that ET 1 was extensively distributed among all host species analyzed. From the viewpoint of biotechnological application, it will be important to determine if strains represented by the highly predominant ET 1 could be more efficient in promoting growth of the host plants by either involving indoleacetic acid (17) or supplying nitrogen (5, 51), or both, in comparison to other lineages, or if ET 1 is simply a highly "successful" lineage adapted to different host species.

Considering the apparent wide capacity of ET 1 to colonize sucrose-rich host plants, it will be interesting to determine this ability of ET 1 strains in other important sugar producer plants such as sugar beet (*Beta vulgaris*).

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ANEXO III.

Artículo "*Coffea arabica* L., a new host plant for *Acetobacter diazotrophicus* and isolation of other Nitrogen-fixing acetobacters".

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Coffea arabica L., a New Host Plant for *Acetobacter diazotrophicus*, and Isolation of Other Nitrogen-Fixing Acetobacteria

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***Acetobacter diazotrophicus* was isolated from coffee plant tissues and from rhizosphere soils. Isolation frequencies ranged from 15 to 40% and were dependent on soil pH. Attempts to isolate this bacterial species from coffee fruit, from inside vesicular-arbuscular mycorrhizal fungi spores, or from mealybugs (*Planococcus citri*) associated with coffee plants were not successful. Other acid-producing diazotrophic bacteria were recovered with frequencies of 20% from the coffee rhizosphere. These N_2-fixing isolates had some features in common with the genus *Acetobacter* but should not be assigned to the species *Acetobacter diazotrophicus* because they differed from *A. diazotrophicus* in morphological and biochemical traits and were largely divergent in electrophoretic mobility patterns of metabolic enzymes at coefficients of genetic distance as high as 0.950. In addition, these N_2-fixing acetobacteria differed in the small-subunit rRNA restriction fragment length polymorphism patterns obtained with *Eco*RI, and they exhibited very low DNA-DNA homology levels, ranging from 11 to 15% with the *A. diazotrophicus* reference strain PAl 5^T. Thus, some of the diazotrophic acetobacteria recovered from the rhizosphere of coffee plants may be regarded as N_2-fixing species of the genus *Acetobacter* other than *A. diazotrophicus*. Endophytic diazotrophic bacteria may be more prevalent than previously thought, and perhaps there are many more potentially beneficial N_2-fixing bacteria which can be isolated from other agronomically important crops.**

Almost 100 bacterial genera, of both the eubacteria and archacobacteria, are capable of fixing N_2 (32). There may exist many more bacterial species or genera which can fix nitrogen since a majority of bacterial species are not presently culturable (31) and the search for diazotrophs in some environments has been relatively limited. Research on N_2-fixing bacteria endophytically associated with sugarcane led to the description of *Acetobacter diazotrophicus*, which is the only known nitrogen-fixing species of acetic acid-producing bacteria (13, 29). Similarly, in the last few years, the genus *Azoarcus* and its various species were described (16, 33), most of them recovered from the roots of Kallar grass (24). These findings suggest that many other endophytic N_2-fixing species may not yet have been described.

Looking for well-known N_2-fixing species and for new diazotrophs associated with previously untested plants or from new environments may provide a better picture not only of the distribution of N_2-fixation ability among bacterial taxa but also of the distribution and diversity of N_2-fixing bacterial populations.

In this work, we report the natural occurrence of diazotrophic acetic acid-producing bacteria in the rhizosphere and in tissues from different cultivars of seed-propagated coffee plants (*Coffea arabica* L.). Microbiological, biochemical, and genetic tests showed that a majority of these bacteria belong to the species *A. diazotrophicus*. We obtained evidence that strongly supports the hypothesis that some of the strains could represent new N_2-fixing species of the genus *Acetobacter*.

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MATERIALS AND METHODS

Locations and coffee cultivars. Coffee plant varieties grown in nurseries or under field conditions were collected from diverse geographic regions of Mexico up to 750 km apart. The origins of samples and the coffee varieties analyzed are summarized in Table 1.

Media and cultural conditions. N-free semisolid LGI medium supplemented with sugarcane juice at pH 4.5 (7) and cycloheximide (150 mg/liter) was used for enrichment culturing of N_2-fixing acetobacters. For isolation and culturing, acetic acid LGI agar plates supplemented with yeast extract (50 mg/liter) and cycloheximide (150 mg/liter) and potato agar plates with 10% cane sugar were used (7). N_2-fixing acetobacters were grown at 29°C in SYP medium (6) for all other assays.

Isolation. Care was taken to keep rhizosphere soil intact around the root. Later, the root samples were rinsed three times in sterile distilled water. Separately, coffee root and stem pieces were immersed in 1% chloramine T for 5 min and treated as described previously (11). Root and stem samples were macerated in a blender, and supernatant aliquots (100 μl) were placed in vials containing 5 ml of N-free semisolid LGI medium (7). Other vials were inoculated with 100-μl aliquots from a 1/10 (wt/vol) rhizosphere soil suspension. Also, five samples (10 g each) of ripening fruit from *Coffea arabica* cv. Garnica collected in the coffee-growing region of Huitzilan, Puebla State, Mexico, were surface sterilized and treated as mentioned above for root and stem samples. In attempts to recover *A. diazotrophicus* from inside vesicular-arbuscular mycorrhizal (VAM) fungal spores, 100 g of eight rhizosphere soil samples (four from Huitzilan and four from Tapachula, Chiapas, Mexico) was sieved and at least 60 VAM spores were isolated from each soil sample by the method described by Gerdemann and Nicolson (12). The VAM spores were surface sterilized with 1% chloramine T for 5 min and then washed four times with sterile distilled water. Spores without apparent damage were manually crushed and placed in vials containing N-free semisolid LGI medium as reported previously (23). In addition, 50 adult mealybugs identified as *Planococcus citri* were analyzed for N_2-fixing acetobacters. These were collected from aerial parts of coffee plants, cultivar Caturra, growing in fields at Atoyac, Guerrero State, Mexico. Groups of 10 insects were rinsed with 0.01% (vol/vol) Tween 20 in 10 mM $MgSO_4 \cdot 7H_2O$ until the liquid was clear. Insects were macerated in 1.0 ml of 10 mM $MgSO_4 \cdot 7H_2O$, and 100-μl

TABLE 1. Isolation frequencies of *A. diazotrophicus* recovered from coffee plant cultivars

Location	Cultivar	Plant age	pH of soil	Isolation (%)		
				Rhzp[a]	Root	Stem
Huitzilan, Puebla	Garnica	5 yr[b]	4.07	40.0	40.0	0.0
	Garnica	2 yr[b]	6.27	0.0	0.0	0.0
Xicotepec, Puebla	Catuai	2 mo[c]	4.74	ND[d]	0.0	20.0
	Catuai	6 mo[b]	4.00	ND	20.0	0.0
Atoyac, Guerrero	Caturra	1 yr[b]	3.64	15.0	20.0	0.0
	M. Novo	1 yr[c]	6.20	0.0	0.0	0.0
Tapachula, Chiapas	Caturra	5 mo[c]	5.40	30.0	0.0	0.0
	Caturra	5 mo[c]	5.80	20.0	0.0	0.0
	Caturra	3 mo[c]	5.30	40.0	0.0	0.0

[a] Rhzp, rhizosphere (soil shaken off roots).
[b] Coffee plants growing under field conditions.
[c] Coffee plants growing in a nursery.
[d] ND, not determined.

aliquots were inoculated into media for isolation of *A. diazotrophicus* as described previously (5).

Vials of inoculated N-free semisolid LGI medium were incubated at 30°C for 7 days. Thereafter, vials were replicated under the same conditions and assayed for acetylene reduction activity as described previously (21). Nitrogenase-positive vials with a yellow surface pellicle were streaked onto acetic LGI agar plates and incubated at 30°C. After 5 to 7 days, acid-producing dark-orange colonies suggested the presence of *A. diazotrophicus* (7). Colonies were streaked on potato agar plates to verify culture purity. In addition, atypical acid-producing isolates (referred to in the text as DOR and APL isolates) were also recovered from coffee rhizosphere samples from Tapachula. These isolates did not exhibit growth typical of *A. diazotrophicus* on LGI agar plates. DOR isolates were similar in their dark-orange color but formed very irregular smooth flat colonies. In addition, while *A. diazotrophicus* colonies are initially white and later become yellow-orange, DOR isolates are always orange. APL isolates showed a liquid-like appearance on the first days, but after 5 days, the isolates became dry and took on a yellowish color. One non-acid-producing mucoid strain (designated CFN-Cf 56) was also isolated due to its predominant growth on an LGI agar plate. This strain was selected based on its colony morphology, which was similar to that of a spontaneous, non-acid-producing mutant that was obtained from *A. diazotrophicus* SRT4 (1).

Identification. Isolate identification was based on colony morphology in culture media, on biochemical tests, and on genetic characteristics reported for *A. diazotrophicus* (5–7, 13). *A. diazotrophicus* PAl 5^T (ATCC 49037), kindly provided by J. Döbereiner, and UAP 5560, analyzed previously (6, 11), were used as controls.

MLEE. Each isolate was grown for 36 h in 40 ml of SYP medium and harvested by centrifugation; pellets were suspended and treated as described previously (6). Starch gel electrophoresis and selective staining of metabolic enzymes were done as described before (25). The analyzed enzymes were the same ones used in a previous study (5) and were assayed under the same conditions. Distinct combinations of alleles for 12 enzyme loci (multilocus genotypes) were designated as different electrophoretic types (ETs) (25). *A. diazotrophicus* strains (CFNE 501, CFNE 550, PAl 5^T, PAl 3, 1772, PSP 22, and PRC 1), corresponding to the reported seven ETs (5), were included as references in multilocus enzyme electrophoresis (MLEE) assays to determine the genetic relationships of coffee plant-associated isolates and *A. diazotrophicus*.

Total DNA isolation, DNA restriction, and filter blot hybridization. Total DNA was isolated as described previously by Ausubel et al. (3). DNA was digested with *Eco*RI, and restriction fragments were electrophoresed in vertical 1.0% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8]) at 40V for 13 h at 4°C. Total DNA digests were transferred from gels to nylon filters by the Southern procedure as described before (6). The restriction fragment-length polymorphism (RFLP) patterns of the *nifHDK* genes were determined by hybridization with a *Hin*dIII-*Hin*dIII 4.3-kb fragment containing the *nifHDK* genes from *A. diazotrophicus* UAP 5560 obtained from pUC19 derivative pNHAd4 (unpublished results). DNA-DNA homology was based on relative levels of hybridization to ^{32}P-labelled DNA from strain PAl 5^T. Amounts of DNA in gels were quantified as described before (5). Autoradiography was performed at −70°C for 24 h; filter lanes were cut and counted with a Beckman scintillation counter. The percentage of total hybridization was calculated for each strain tested. Hybridization patterns of small-subunit (SSU) ribosomal DNA (rDNA) genes were analyzed as described before (5), but in this study, total DNA also was digested with restriction enzymes *Sph*I and *Nco*I. Genomic DNA from coffee plant-associated isolates and from strains PAl 5^T and UAP 5560 of *A. diazotrophicus* were hybridized with an *Escherichia coli* SSU rRNA gene internal fragment from vector pKK3535 (4) corresponding to nucleotides 80 to 653. ^{32}P-labelled probes were prepared by nick translation.

SSU rDNA sequence alignment. To search discriminative restriction sites in the SSU rRNA genes for distinguishing *Acetobacter* from other bacteria, we aligned 11 reported SSU rDNA sequences of different strains of the family *Acetobacteraceae* and 29 sequences of strains from other members of the α subclass of the class *Proteobacteria* (α-*Proteobacteria*) with GCG software version 8.1-UNIX (Genetics Computer Group, Madison, Wis.). GenBank accession numbers for SSU rDNA sequences aligned are shown in Table 2.

RESULTS

Isolation. Typical yellow surface pellicles of nitrogen-fixing *Acetobacter* were observed in N-free LGI medium vials inoculated with rhizosphere soil, blended roots, and stems from different coffee plant varieties grown in various geographical areas of Mexico. On LGI agar plates, dark-orange colonies typical of *A. diazotrophicus* were observed (Fig. 1). Isolation frequencies from the rhizosphere, inside of roots, or stems ranged from 15 to 40% in plants grown in acid soils (Table 1). Additionally, from some rhizosphere samples, we recovered acid-producing DOR and APL isolates from LGI medium vials

TABLE 2. GenBank accession numbers used for the SSU rDNA sequence alignments

Species	Strain	Accession no.
Acetobacter pasteurianus	LMD 22.1	X71863
Acetobacter aceti	DSM 3508	X74066
Acetobacter liquefaciens	LMG 1382	X75617
Acetobacter diazotrophicus	PAl 5^T	X75618
Acetobacter xylinum	NCIB 11664	X75619
Acetobacter hansenii	NCIB 8746	X75620
Acetobacter europaeus	DSM 6160	Z21936
Gluconobacter oxydans	DSM 3503	X73820
Gluconobacter asaii	LMG 1390	X80165
Gluconobacter cerinus	LMG 1368	X80775
Gluconobacter frateurii	LMG 1365	X82290
Acidomonas methanolica	LMG 1668[a]	X77468
Acidiphilium sp.	C-1	D30769
Acidiphilium aminolytica	101	D30771
Acidiphilium angustum	ATCC 35903	D30772
Acidiphilium cryptum	ATCC 33463	D30773
Acidiphilium facilis	ATCC 35904	D30774
Acidiphilium organovorum	ATCC 43141	D30775
Acidiphilium rubrum	ATCC 35905	D30776
Acidiphilium sp.	St 1-5	D86508
Acidiphilium sp.	St 1-7	D86509
Rhodopila globiformis	DSM 161	D86513
Rhodopila globiformis	ATCC 7950	M59066
Rhodospirillum sp.	MT-SP-3	D12703
Rhizobium meliloti	IAM12611	D14509
Rhizobium leguminosarum	IAM12609	D14513
Rhodopseudomonas sp.	IL-245	D15063
Rhodobacter capsulatus	ATCC 11166	D16428
Rhodospirillum rubrum	ATCC 11170	D30778
Beijerinckia indica	ATCC 9039	M59060
Caulobacter sp.	MCS 6	M83811
Hyphomonas sp.	MHS 3	M83812
Hyphomicrobium vulgare	MC-750	X53182
Roseobacter litoralis	ATCC 49556	X78312
Azospirillum lipoferum	ATCC 29708	X79729
Azospirillum irakense	103312	X79737
Azospirillum brasilense	Sp 7	X79739
Azospirillum amazonense	Y2	X79742
Xanthobacter flavus	JW/KR-E1	X94206
Pedomicrobium manganicum	ACM 3038	X97691

[a] Substrain MB 58.

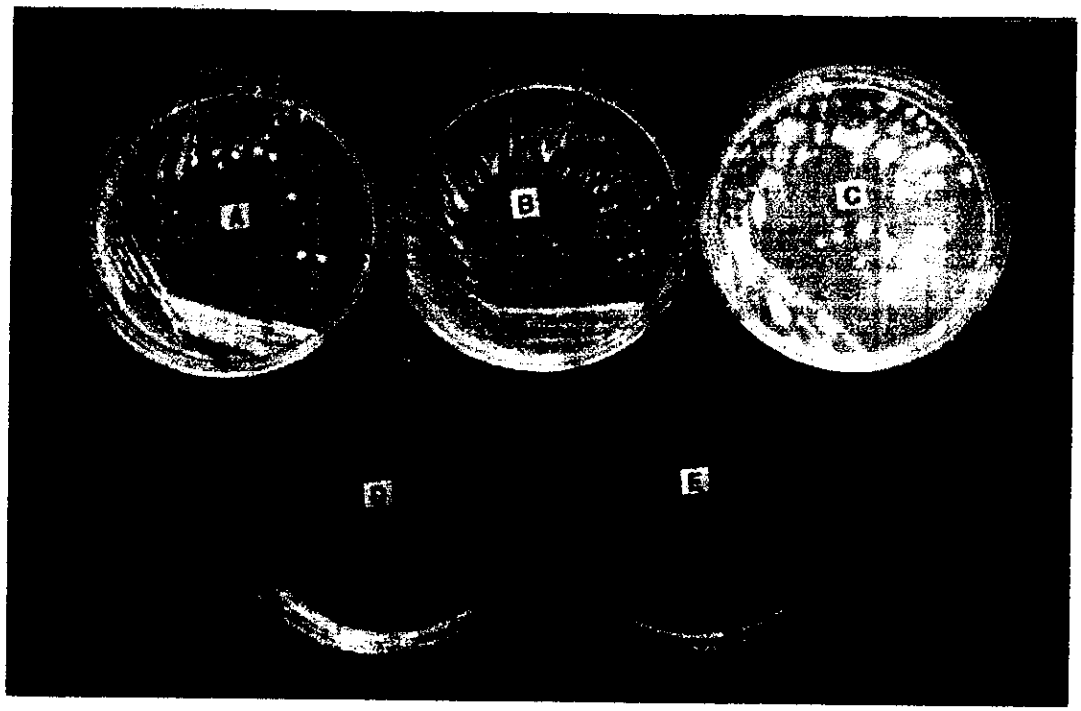

FIG. 1. Colony morphologies of N_2-fixing acetobacters after 7 days at 29°C on LGI agar plates. (A and B) *A. diazotrophicus* PAl 5^T (A) and CFN-Cf 50 (B); (C) DOR isolate, strain CFN-Cf 55; (D) APL isolate, strain UAP-Cf 60; (E) mucoid strain CFN-Cf 56. The green color in LGI agar plates was turned to yellow by acid-producing isolates.

with yellow surface pellicles. These isolates reduced acetylene in pure culture but had clearly different morphologies from that of *A. diazotrophicus* on LGI agar plates (Fig. 1). These DOR and APL isolates were recovered from two rhizosphere samples (collected in Tapachula) with isolation frequencies of 20%. Strain CFN-Cf 56, which does not produce acid on LGI agar plates, was the only mucoid isolate recovered (Fig. 1).

No bacteria corresponding to the descriptions given above were isolated from coffee plants growing at a pH higher than 6.0 nor from coffee fruit, VAM spores, or mealybugs (*P. citri*).

MLEE and genetic relationships. The origins of the coffee-associated N_2-fixing isolates are shown in Table 3. The genetic relationships among the N_2-fixing isolates associated with coffee plants and *A. diazotrophicus* strains recovered from known hosts are illustrated by the dendrogram shown in Fig. 2. Thirteen distinct ETs were identified among N_2-fixing coffee iso-

TABLE 3. Origins of representative N_2-fixing bacteria recovered from the coffee environment

MLEE division (ET)[a]	Type of isolate	Strain designation	Isolate recovered from:	Plant age	Cultivar	Location
I (1)	*A. diazotrophicus*	CFN-Cf13	Stem tissue	2 mo[b]	Catuai	Xicotepec, Puebla
I (11)	*A. diazotrophicus*	CFN-Cf50	Root tissue	6 mo[c]	Catuai	Xicotepec, Puebla
I (1)	*A. diazotrophicus*	UAP-Cf29	Rhizosphere	1 yr[c]	Caturra	Atoyac, Guerrero
I (9)	*A. diazotrophicus*	CFN-Cf52	Root tissue	1 yr[c]	Caturra	Atoyac, Guerrero
I (8)	*A. diazotrophicus*	UAP-Cf01	Rhizosphere	5 yr[c]	Garnica	Huitzilan, Puebla
I (8)	*A. diazotrophicus*	UAP-Cf05	Root tissue	5 yr[c]	Garnica	Huitzilan, Puebla
I (12)	*A. diazotrophicus*	UAP-Cf51	Rhizosphere	5 mo[b]	Caturra	Tapachula, Chiapas
I (14)	*A. diazotrophicus*	UAP-Cf53	Rhizosphere	3 mo[b]	Caturra	Tapachula, Chiapas
I (10)	*A. diazotrophicus*	UAP-Cf58	Rhizosphere	5 mo[b]	Caturra	Tapachula, Chiapas
I (13)	NAP[d]	CFN-Cf56	Rhizosphere	3 mo[b]	Caturra	Tapachula, Chiapas
III (15)	APL[e]	UAP-Cf59	Rhizosphere	5 mo[b]	Caturra	Tapachula, Chiapas
III (16)	APL	CFN-Cf60	Rhizosphere	3 mo[b]	Caturra	Tapachula, Chiapas
IV (17)	DOR[f]	CFN-Cf55	Rhizosphere	3 mo[b]	Caturra	Tapachula, Chiapas
IV (18)	DOR	UAP-Cf57	Rhizosphere	5 mo[b]	Caturra	Tapachula, Chiapas
V (19)	SAd[g]	CFN-Cf54	Rhizosphere	5 mo[b]	Caturra	Tapachula, Chiapas

[a] Divisions and ETs were based on MLEE assays. More isolates included in divisions I and III to V were recovered, but only one of the many isolates recovered from each plant or rhizosphere sample was designated as a strain.
[b] Coffee plants growing in a nursery.
[c] Coffee plants growing under field conditions.
[d] NAP, non-acid-producing isolate.
[e] APL, acid-producing liquid isolate.
[f] DOR, dark-orange isolate.
[g] SAd, isolate with colonial features similar to those of *A. diazotrophicus*.

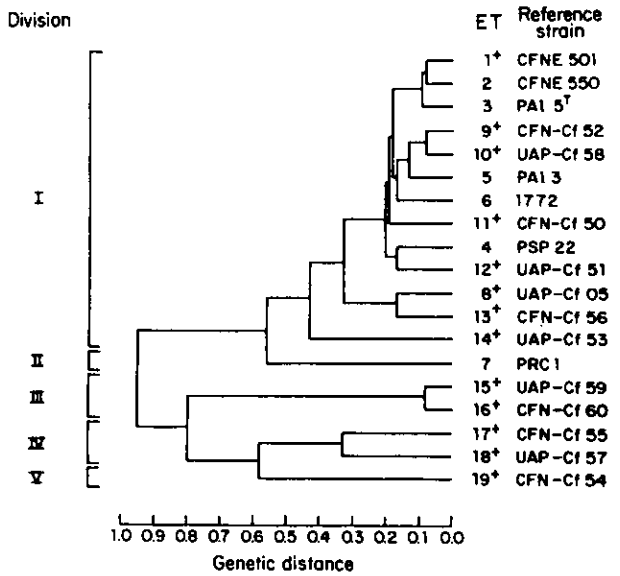

FIG. 2. Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from well-known hosts and N_2-fixing acetobacters associated with coffee plants. A plus after the ET number indicates that the ET represents only coffee plant-associated nitrogen-fixing acetobacters, except for ET 1, which includes reported reference strains as well.

lates (multilocus genotype data are available upon request). Division I, with a genetic distance of 0.430, included six previously identified ETs (ET 1 to ET 6) (5) and six new closely related ETs (ET 8 to ET 12 and ET 14) from coffee-associated *A. diazotrophicus* isolates. In addition, division I included ET 13, which corresponds to an isolate (CFN-Cf 56) with no typical features of *A. diazotrophicus*. Moreover, isolates recovered from both the rhizosphere (e.g., strain UAP-Cf 29) and the inside of coffee plants (e.g., strain CFN-Cf 13) were identical to strains of *A. diazotrophicus* belonging to ET 1, previously identified (5, 6) as the predominant ET (e.g., UAP 5560 and CFNE 501) of the species. Division II contained only ET 7, a genetically distant group previously identified (5) among *A. diazotrophicus* strains isolated from sugarcane and *Pennisetum purpureum* in Brazil. Divisions III, IV, and V, which included ETs 15 to 19, diverged largely at a genetic distance of 0.950 from divisions I and II. Division III (ETs 15 and 16) contained only APL isolates, while division IV (ETs 17 and 18) included DOR isolates and division V (ET 19) grouped isolates with colonial features similar to those of *A. diazotrophicus* on acetic LGI agar plates.

Identification. Many isolates recovered from the inside of coffee plants and from the rhizosphere of these plants were identified as belonging to the species *A. diazotrophicus* on the basis of reported characteristics (5, 6, 7, 13) such as growth features on culture media, biochemical tests, and results of genetic approaches (Tables 4 and 5). Other isolates such as the mucoid strain CFN-Cf 56 and the DOR and APL strains differed from *A. diazotrophicus* in various phenotypic characteristics (Table 4 and carbon usage data not shown). Nevertheless, these isolates were able to grow at pH 5, oxidize ethanol to acetic acid in neutral and acid conditions, and oxidize acetate and lactate to CO_2 and H_2O (Table 4), phenotypic features which are considered (8, 29) fundamental for the identification of the genus *Acetobacter*.

Genetic characteristics. Total *Eco*RI DNA digests from coffee isolates, including those with different colony morpholo-

TABLE 4. Phenotypic characteristics of N_2-fixing acetic acid bacteria isolated from the coffee plant environment[a]

Characteristic	I[b]						III		IV		V
	PAI 5[Tr]	UAP 5560[c]	CFN-Cf 13	UAP-Cf 05	CFN-Cf 52	CFN-Cf 56	UAP-Cf 59	CFN-Cf 60	CFN-Cf 55	UAP-Cf 57	CFN-Cf 54
Gram stain	−	−	−	−	−	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidation of ethanol to acetic acid	+	+	+	+	+	+[d]	+	+	+	+	+
Oxidation of glucose to acetic acid	+	+	+	+	+	+	+	+	+	+	+
Oxidation of acetic acid to CO_2 and H_2O	+	+	+	+	+	+	+	+	+	+	+
Oxidation of lactate to CO_2 and H_2O	+	+	+	+	+	+	+	+	+	+	+
Water-soluble brown pigments on GYC[e]	+	+	+	+	−	−	−	−	−	−	−
Dark-orange colonies on LGI plates	+	+	+	+	+	−	−	−	−	−	−
Dark-brown colonies on potato agar with 10% sugar	+	+	+	+	+	−	−	−	−	−	−
Brownish colonies on potato agar with 10% sugar	−	−	−	−	−	−	−[f]	−[f]	+	+	+
Growth with 30% D-glucose	+	+	+	+	+	+	+	+	+	+	+
Growth with 30% sucrose	+	+	+	+	+	+	+	+	+	+	+
Yellow surface pellicle formation and pH below 3 in N-free semisolid LGI medium	+	+	+	+	+	−	+	+	+	+	+
C_2H_2 reduction activity (N_2 fixation)	+	+	+	+	+	+	+	+	+	+	+

[a] Phenotypic characteristics were positive (+) or negative (−) for each strain.
[b] Divisions based on MLEE assays.
[c] *A. diazotrophicus* strains recovered from sugarcane used as controls.
[d] Oxidation was observed up to day 4.
[e] Cream-colored colonies, but producing a brownish liquid pigment.
[f] GYC, 5% D-glucose–1% yeast extract–3% $CaCO_3$–2.5% agar (8).

TABLE 5. Genetic characteristics of some N_2-fixing acetobacters recovered from the coffee plant environment[a]

MLEE division[a] (ET)[a]	Type of isolate[b]	Reference strain	Sizes (kb)[c]		DNA-DNA homology (%)[d]
			nifHDK genes	SSU rDNA genes	
I (3)	*A. diazotrophicus*	PAI 5[T][e]	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	100.0
I (1)	*A. diazotrophicus*	UAP 5560[e]	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	104.0
I (8)	*A. diazotrophicus*	UAP-Cf 05	9.0, 3.1, 1.25	9.3, 3.6, 2.3, 1.6	ND[f]
I (9)	*A. diazotrophicus*	CFN-Cf 52	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	83.0
I (10)	*A. diazotrophicus*	UAP-Cf 58	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	78.0
I (11)	*A. diazotrophicus*	CFN-Cf 50	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	96.0
I (12)	*A. diazotrophicus*	UAP-Cf 51	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	72.0
I (14)	*A. diazotrophicus*	UAP-Cf 53	9.0, 2.0, 1.25	9.3, 3.6, 2.3, 1.6	77.0
I (13)	NAP	CFN-Cf 56	7.6, 3.5, 1.20, 1.0	9.3, 3.6, 2.3, 1.6	30.0
III (15)	APL	UAP-Cf 59	Not detected	8.6, 3.9, 3.6, 1.6	12.0
III (16)	APL	CFN-Cf 60	Not detected	8.6, 3.9, 3.6, 1.6	15.0
IV (17)	DOR	CFN-Cf 55	9.0, 2.0, 1.20	9.7, 3.6, 1.6	14.0
IV (18)	DOR	UAP-Cf 57	9.0, 2.0, 1.20	9.7, 3.6, 1.6	15.0
V (19)	SAd	CFN-Cf 54	6.6, 2.1, 1.15	9.7, 3.6, 2.8, 1.6	11.0

[a] Divisions and ETs were based on MLEE assays.
[b] Types described in Table 3, footnotes *d*, *e*, *f*, and *g*.
[c] Bands from total *Eco*RI DNA fingerprints hybridized as described in Materials and Methods.
[d] Homology to the control strain PAI 5[T].
[e] *A. diazotrophicus* strains recovered from sugarcane used as controls.
[f] ND, not determined.

gies, were hybridized to *A. diazotrophicus nifHDK* genes (Fig. 3). Three common hybridizing bands were observed for representative isolates of the 6 ETs from division I (Table 5), as reported previously (5, 6). In addition, isolates represented by strain UAP-Cf 05 (division I) and isolates grouped in division IV (e.g., CFN-Cf 55 and UAP-Cf 57) showed bands that differed from each other slightly in size (Table 5). Strain CFN-Cf 54 (division V) and the mucoid strain CFN-Cf 56 showed a more variable pattern of the *nifHDK* genes. No hybridizing bands were observed under stringent hybridization conditions with APL strains from division III (Fig. 3), even though pure cultures of these isolates were capable of reducing acetylene. This result seems to indicate that structural nitrogenase genes from APL isolates are largely divergent from *A. diazotrophicus nifHDK* genes.

RFLP analysis of *Eco*RI DNA digests from coffee plant-associated isolates showed four distinct hybridization patterns to SSU rRNA genes (Fig. 4). Among the patterns obtained, two common hybridizing bands (3.6 and 1.6 kb) were observed. All isolates of division I showed the same pattern of hybridization (Table 5) as that observed previously in all *A. diazotrophicus* strains analyzed (5). N_2-fixing *Acetobacter* strains diverging at a large genetic distance from divisions I and II, according to the MLEE assays, presented different SSU rRNA hybridization patterns (Fig. 4 and Table 5). Isolates grouped in division IV did not have the 2.3-kb band which seems to correspond to the 3.9- and 2.8-kb bands observed in the strains from divisions III and V, respectively.

From the SSU rDNA sequence analysis, we inferred that Southern hybridization with a SSU rDNA probe of *Sph*I-digested genomic DNA could be helpful in distinguishing members of the family *Acetobacteraceae* from other α-*Proteobacteria* (Fig. 5) and that *Nco*I digests could be used to distinguish the genera *Gluconobacter* and *Acetobacter* from *Acidiphilium* and *Rhodopila* (Fig. 5) (26). The majority of *Acetobacteraceae* spe-

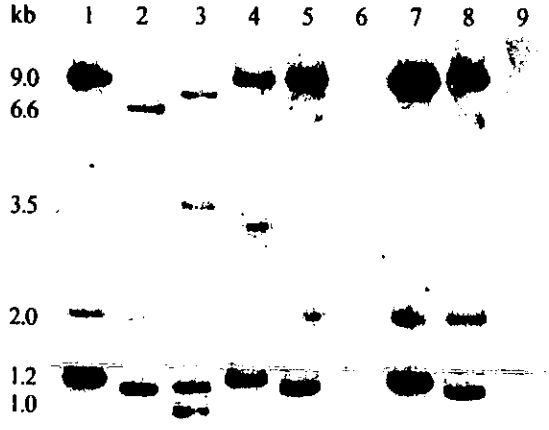

FIG. 3. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with the *nifHDK* probe of *A. diazotrophicus* UAP 5560. Lanes: 1, strain UAP 5560 used as a control; 2 to 9, coffee plant-associated nitrogen-fixing strains CFN-Cf 54 (lane 2), CFN-Cf 56 (lane 3), UAP-Cf 05 (lane 4), CFN-Cf 57 (lane 5), UAP-Cf 59 (lane 6), UAP-Cf 13 (lane 7), CFN-Cf 55 (lane 8), and CFN-Cf 60 (lane 9).

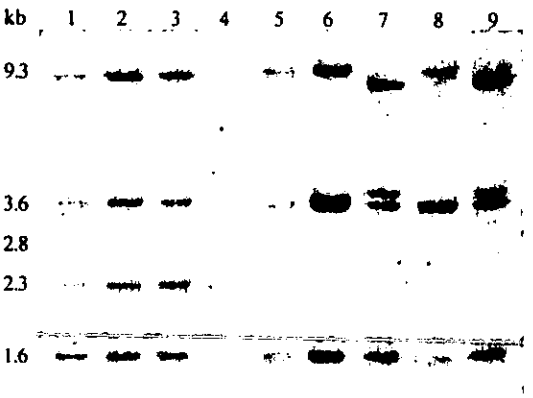

FIG. 4. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with an internal 16S rDNA probe of *E. coli*. Lanes: 1, strain UAP 5560 used as a control; 2 to 9, coffee-associated nitrogen-fixing strains UAP-Cf 13 (lane 2), UAP-Cf 05 (lane 3), CFN-Cf 56 (lane 4), CFN-Cf 54 (lane 5), UAP-Cf 57 (lane 6), UAP-Cf 59 (lane 7), CFN-Cf 55 (lane 8), and CFN-Cf 60 (lane 9).

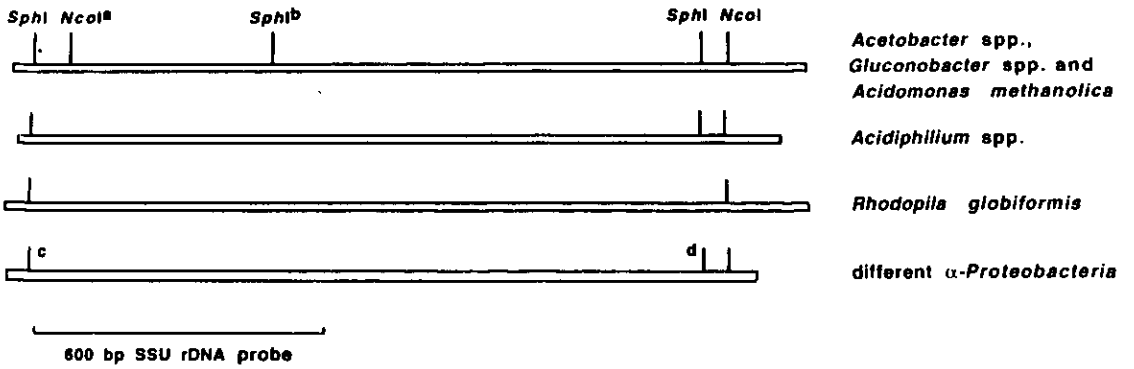

FIG. 5. Diagrammatic representation of distinctive restriction sites *Sph*I and *Nco*I of SSU rRNA in *Acetobacteraceae* and phenotypically related bacteria. a, site not present in *G. oxydans* DSM 3503; b, site exclusively present in the *A. diazotrophicus* PAl 5^T sequence but not detected after Southern hybridization; c, site present in *Azospirillum lipoferum* ATCC 29708 and *Azospirillum amazonense* Y2; d, of 17 analyzed sequences, this site exclusively present in *Rhizobium meliloti* IAM 12611, *Rhizobium leguminosarum* IAM 12609, *Caulobacter* sp. strain MCS 6, *Hyphomonas* sp. strain MHS 3, and *Xanthobacter flavus* JW/KR-E1.

cies, including *Acidomonas methanolica*, have two internal *Sph*I sites in their SSU rDNA, except for *A. diazotrophicus* PAl 5^T (accession number X75618), which supposedly has an extra *Sph*I site at base 485 as deduced from the reported sequence (Fig. 5). *Rhodopila globiformis* (accession numbers D86513 and M59066) lacks one of the *Sph*I sites. From the analysis of the *A. diazotrophicus* PAl 5^T SSU rDNA sequence (26), we expected to observe one hybridizing band of 450 bp with the probe used when the DNA was digested with *Sph*I. However, only one SSU rRNA hybridizing band of 1.3 kb was observed in *A. diazotrophicus* PAl 5^T and UAP 5560. This band was conserved in all coffee plant-associated isolates. These conflicting results may be explained if the *A. diazotrophicus* sequence has an error at the *Sph*I site. If such were the case, then the *Acetobacteraceae* and *Acidiphilium* spp. would have only two *Sph*I conserved sites. *Gluconobacter* and *Acetobacter* SSU rDNA are characterized by two *Nco*I restriction sites. However, all *Acidiphilium* and *Rhodopila* species and *Gluconobacter oxydans* lack the *Nco*I restriction site at the base corresponding to nucleotide 110 of *A. diazotrophicus*. The rest of the α-*Proteobacteria* analyzed lack at least one site for each restriction enzyme. Genomic DNA from the strains recovered from the coffee plant environment, digested with *Nco*I and hybridized to the same SSU rRNA internal gene fragment, showed the expected 1.24-kb band (26) (data not shown).

The results of the DNA-DNA homology assays are shown in Table 5. The six strains of N_2-fixing acetobacters corresponding to division I (except strain CFN-Cf 56) analyzed were related to *A. diazotrophicus* PAl 5^T with DNA homology values of 72 to 96%, with a mean DNA homology of 81%. This value was consistent with the values of 86 and 84% reported previously (5, 13) for *A. diazotrophicus* strains recovered from sugarcane and other known hosts. The mucoid strain CFN-Cf 56 exhibited only 30% DNA homology to strain PAl 5^T. APL isolates (MLEE division III) and DOR acetobacters from division IV and strains from division V exhibited very low DNA homology levels, ranging from 11 to 15% with reference strain PAl 5^T.

DISCUSSION

It is considered that "the isolation of acetic acid bacteria and their assignment to either the genus *Acetobacter* or *Gluconobacter* generally pose few problems" (29). According to Swings (29), gram-negative or gram-variable aerobic bacteria that oxidize ethanol to acetic acid in neutral or acid media are candidates for the family *Acetobacteraceae*. This family is divided into the genera *Gluconobacter*, which includes three species, and *Acetobacter*, in which seven species have been identified (29). Only the species *A. diazotrophicus* is capable of fixing N_2 (13). On the basis of these and other phenotypic features used for a satisfactory identification (29), we considered that the diazotrophic isolates recovered from the coffee plant environment belong to the family *Acetobacteraceae*. Phenotypic identification was confirmed by the SSU rRNA genes obtained with total DNA digested with *Nco*I and *Sph*I (data not shown). Moreover, we have considered it suitable to assign these N_2-fixing isolates to the genus *Acetobacter* because they were capable of oxidizing ethanol, first to acetic acid and then further to CO_2 and H_2O (overoxidation of ethanol), which is the main feature of the genus (8, 29). Other differential phenotypic characteristics analyzed (Table 4) were in agreement with descriptions for this genus (8, 29). By taking into account the differential phenotypic features at the species level (8, 29) and with support from the MLEE assays and the molecular characteristics reported previously, such as hybridization patterns of *nifHDK* genes and of SSU rDNA genes (5, 6) as well as DNA-DNA homology experiments, a majority of the N_2-fixing *Acetobacter* isolates (all strains from division I, excluding CFN-Cf 56) recovered from rhizosphere soil and from inside tissues of coffee plants were considered to belong to the species *A. diazotrophicus*. Although *A. diazotrophicus* strains were reported to form water-soluble brown pigments on GYC medium (7), some of the *A. diazotrophicus* strains (CFN-Cf 52, UAP-Cf 51, Cf 53, and Cf 58) recovered from the coffee plant environment did not produce them (Table 4). However, water-soluble brown pigment production is not a typical feature of the genus *Acetobacter* but rather of the genus *Frateuria* (30). Thus, the *A. diazotrophicus* isolates not producing water-soluble brown pigments could be considered more typical acetobacters.

A number of *Acetobacter* isolates recovered from the coffee plant rhizosphere, capable of fixing N_2 under microaerobic conditions, should not be assigned to the species *A. diazotrophicus* because remarkable differences were observed. We propose that the strains corresponding to ETs included in divisions III, IV, and V may be regarded as different N_2-fixing species of the genus *Acetobacter*. This is based on the fact that all of these isolates were easily differentiated from *A. diazotrophicus* by several morphological and biochemical traits, including the electrophoretic mobility patterns of metabolic enzymes, rendering coefficients of genetic distance as high as 0.950.

Furthermore, these *Acetobacter* isolates differed in SSU rRNA RFLP patterns, and they had a very low level of DNA homology with *A. diazotrophicus* PAl 5^T. These data are strong evidence to designate other diazotrophic species of the genus *Acetobacter*, but more N_2-fixing isolates from other coffee-producing areas of Mexico have to be isolated to provide an extended phenotypic and genetic analysis useful for taxonomic validation of a new species. This is specially true for strain CFN-Cf 56, which is a unique isolate with peculiar characteristics. For instance, on the basis of the MLEE data and SSU rRNA RFLP patterns, the strain CFN-Cf 56 should be regarded as belonging to the species *A. diazotrophicus*. However, on the basis of DNA-DNA homology values, this strain may be considered a new nitrogen-fixing species of the genus *Acetobacter*. Nevertheless, plasmid differences could account for the low DNA-DNA homology values between strain CFN-Cf 56 and strain PAl 5^T.

Natural habitats of acetic acid bacteria are sugar and alcohol solutions, with flowers and many fruits being excellent habitats (29). *A. diazotrophicus*, an endophytic bacterial species, occurs predominantly in vegetatively propagated plants (9). It has been recovered from inside tissues of sucrose-accumulating plants such as sugarcane (10, 11, 19), from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv. Cameroon, and from sweet potato stems and roots (9) as well as from different genera of mealybugs associated with sugarcane plants (2, 5). This species has not been recovered from other plants nor from nonrhizosphere soils collected from sugarcane fields or other sites (9, 19). However, *A. diazotrophicus* was detected in sugarcane rhizosphere soil by the indirect enzyme-linked immunosorbent assay method (20).

In this study, *A. diazotrophicus* was isolated mainly from coffee plant rhizosphere soils but also, in lower frequencies, from surface-sterilized stems and roots of coffee plants. Our results strongly contrast those of previous reports in which *A. diazotrophicus* isolation from the sugarcane rhizosphere was a rare event. The occurrence of VAM fungus species associated with coffee plants (28) could explain the frequent isolation of *A. diazotrophicus* from the rhizosphere since this bacterial species has been reported to occur inside VAM fungal spores (23), and these were not discarded from the soil inoculated into the culture medium. However, our results did not support the former possibility because we were unable to recover *A. diazotrophicus* from VAM spores. The recovery of N_2-fixing acetobacters from the rhizosphere, we suspect, could be in relation to the organic matter content present in the rhizosphere of coffee plants. While sugarcane is burned off before cutting, eliminating virtually all organic matter originating both from senescent and trash leaves, in coffee-producing areas, the falling fruit and leaves of these trees are largely accumulated in the soil. Perhaps this organic matter could protect bacteria against soil physicochemical factors. In addition, the organic matter degradation by microbial communities will enrich the rhizosphere with carbon (sugar) sources usable by acetobacters. Contrasting with previous results, our data demonstrated that *A. diazotrophicus* is capable of colonizing plants propagated through seeds in addition to plants propagated vegetatively.

Clearly, the distribution of *A. diazotrophicus* is wider than early reports indicated. Genotype ET 1 is extensively distributed, not only among the previously reported hosts (5, 6) but also among coffee plant isolates. Perhaps ET 1 strains have a large colonization capacity that could be related to the presence of a highly conserved plasmid (pAd170) that exists in most ET 1 *A. diazotrophicus* isolates (6). This plasmid has not been observed in isolates corresponding to other ETs (6; unpublished results). pAd170 was also observed in ET 1 isolates recovered both from the rhizosphere and inside coffee plants (data not shown).

Coffee-associated genotypes, except ET 1, were never identified among more than 70 *A. diazotrophicus* strains recovered from previously well-known hosts collected in diverse countries (5, 6). Because isolates of *A. diazotrophicus* recovered from the coffee plant environment are closely related genetically to isolates recovered from sugarcane, the existence of a common lineage is suggested.

It is worth noting that even though the isolation of *A. diazotrophicus* from internal tissues was infrequent, it was usually recovered from coffee plants grown in acid soils. The infrequency of recovery of *A. diazotrophicus* from coffee plant tissues may be related to the difficulties in homogenizing roots and stems, since these plants are highly lignified and very hard to blend. The presence of *A. diazotrophicus* in acid soils suggests that the transmission of this species into coffee plants could be through VAM fungi, as reported for sugarcane plants (22) and *Sorghum bicolor* (17). Also, we considered that transmission of *A. diazotrophicus* could be through mealybugs, as suggested previously (2), or directly into coffee plant fruit, as occurs in pineapple with other acetic acid bacteria (15). Nevertheless, we were not able to recover *A. diazotrophicus* nor any other N_2-fixing acetobacters from coffee plant fruit or mealybugs (*Planococcus citri*). From these results, we may speculate that *A. diazotrophicus* uses root tips and cracks at lateral root junctions to enter the coffee plants, as suggested for sugarcane plants (18).

Our results support the hypothesis that in nature there are many more N_2-fixing bacteria to be identified and also strongly suggest that endophytic diazotrophic bacteria are more prevalent than previously was thought.

Considering the great economic importance of coffee in the world, and the difficulties of obtaining nitrogen fertilizers (14), we consider that coffee-associated N_2-fixing acetobacters may be agronomically important because they could supply part of the nitrogen that the crop requires, as has been suggested in the case of sugarcane with its associated endophytic nitrogen-fixing bacteria.

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ANEXO IV.

Capítulo de libro de congreso "Taxonomy of the family Acetobacteraceae".

TAXONOMY OF THE ACETOBACTERACEAE FAMILY

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The Acetobacteraceae includes acetic bacteria that occur in different vegetal products and colonize the surface and internal parts of plants. According to the SSU rDNA sequences of the currently known members of the α-Proteobacteria, the Acetobacteraceae is located in a branch with close relation to the family Rhodospirillaceae (*Azospirillum* and relatives). As in other α-Proteobacteria, nitrogen fixing strains have been identified in the Acetobacteraceae. N_2-fixing species, like Rhizobia, Rhodobacters and Bradyrhizobia, are located in more distant branches of the α-Proteobacteria (Ludwig et al., 1998).

The taxonomy based on the ribosomal gene sequences of the Acetobacteraceae species has shown almost complete agreement with the traditional taxonomy of this family. The currently recognized genera in the Acetobacteraceae are *Acetobacter* and *Gluconobacter* (Figure 1). Based on the type of prevalent producing ubiquinone and on the SSU rDNA sequence analysis, Yamada et al. (1997) proposed to split the genus *Acetobacter* into two clusters, one with a new genus name *Gluconacetobacter* (*Gluconoacetobacter* [sic]) for those species that synthesize ubiquinone Q_{10}, excepting *A. methanolicus*, and the other, with the former name, *Acetobacter*, for the ubiquinone Q_9-possessing group, with species that show the highest relation with *A. aceti*. From the SSU rDNA tree it is noticeable that the family Acetobacteraceae is composed by different clusters. One of them corresponds to the genus *Gluconobacter*. The other one contains a single species, *A. methanolicus*. Two other clusters are the ones that have been proposed to be relocated to *Gluconacetobacter*, one of that clusters is composed by species related to *A. liquefaciens* and the other one with species related to *A xylinus*. The proposal of the new genus, *Gluconacetobacter*, needs to be corroborated by data from other gene sequences. In *A. diazotrophicus* we have documented the existence of several copies of ribosomal genes (Caballero-Mellado et al., 1995) and recombination events that have been shown in multicopy ribosomal genes in other bacteria may cause distortion in phylogenies based on ribosomal genes.

The only N_2-fixing species recognized so far in this family, *A. diazotrophicus* is located in the same cluster of *A. liquefaciens*. We have isolated and characterized N_2-fixing Acetobacteraceae strains that show phenotypic and SSU rDNA sequence differences with *A. diazotrophicus* (Jiménez-Salgado, 1997; Caballero-Mellado et al., 1999). By SSU rDNA sequence, these strains are located in the same cluster as *A. diazotrophicus* and *A. liquefaciens*. The type strain of one of the new groups, CFN-Cf55^T, shows a SSU rDNA similarity of 98.47 % to *A. diazotrophicus* PAl5^T. The type strain of the other group, CFN-Ca54^T, shows a similarity of 95.25% to *A. diazotrophicus* PAl5^T. The DNA homologies of the strains CFN-Cf 55^T and CFN-Ca54^T with *A. diazotrophicus* PAl5^T are of 19 and 12%, respectively. With the aim of designing SSU rDNA-based oligonucleotides that could be useful in rapid identification of the strains related to CFN-Cf55^T and to CFN-Ca54^T we aligned the known SSU rDNA sequences of the species of Acetobacteraceae. In the SSU rDNA genes there are three regions that could be candidates for designing taxonomic oligonucleotides in this family. We designed primers that differentiate the *A.*

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diazotrophicus, the CFN-Cf55^T- and the CFN-Ca54^T-like strains from all other Acetobacteraceae species described so far.

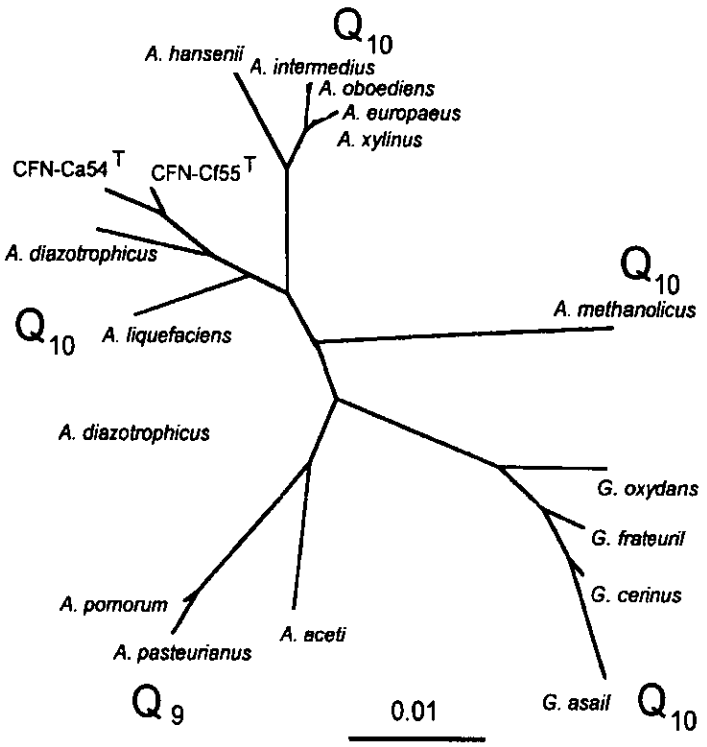

Figure 1 Phylogenetic relationships of the Acetobacteracea family based on SSU rDNA

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ANEXO V.

Capítulo de libro de congreso "Polyphasic taxonomy of nitrogen-fixing bacteria isolated from the rhizosphere of coffee plants".

POLYPHASIC TAXONOMY OF NITROGEN-FIXING ACETIC BACTERIA ISOLATED FROM THE RHIZOSPHERE OF COFFEE PLANTS

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1. INTRODUCTION

Nitrogen fixation has been reported in almost 100 bacterial genera, but new diazotrophic bacteria over the last few years have been described. Because a majority of bacterial species are presently non-culturable and the search for diazotrophs in some environments has been relatively limited, it is recognized that there exist many more N_2-fixing bacteria to be identified.

Studies on N_2-fixing bacteria associated with coffee (*Coffea arabica* L.) plants led us to the isolation of *Acetobacter diazotrophicus* (Jiménez-Salgado et al., 1997), which is the only known N_2-fixing species of acetobacteria described at present (Swings, 1992). In addition, we recovered acid-producing diazotrophic bacteria from the coffee rhizosphere, which shared features with the genus *Acetobacter*. In this study, we showed an extended taxonomic analysis of these N_2-fixing acetobacteria.

2. MATERIALS AND METHODS

Isolates referred to in the text as DOR, APL, and NAP using different phenotypic and genetic approaches were analyzed. The studies were based on colony morphology in culture media, on biochemical tests (Cavalcante and Döbereiner, 1988; De Ley et al., 1984), and on multilocus enzyme polymorphism, on hybridization patterns of 16S rDNA and *nifHDK* genes, as well as DNA-DNA homology experiments, accord-

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ing to procedures described previously (Caballero-Mellado et al., 1995; Gillis et al., 1989). Also, discriminative restriction sites in the 16S rRNA genes were searched for distinguishing *Acetobacteracea* from other bacteria. The isolates were compared by riboprinting of 16S rDNA genes. 16S rDNA genes were PCR-amplified with the primers fD1 and rD1 (Weisburg et al., 1991). The PCR product was restricted with endonucleases *Hha*I, *Msp*I, *Hinf*I, *Sau*3AI, and *Dde*I.

3. RESULTS

Acid-producing DOR and APL isolates form a yellow surface pellicle in N-free LGI semisolid medium and showed acetylene reduction activity (ARA) in pure culture, but clearly differed in colony morphology from that of *A. diazotrophicus* on LGI plates. DOR isolates are similar in their orange color but form irregular smooth flat colonies. APL isolates, after 5 days, became dry and took on yellowish color. NAP isolates form a white surface pellicle in N-free LGI medium and showed ARA. These isolates did not produce acid on LGI plates. Colony morphologies were shown in a previous report (Jiménez-Salgado et al., 1997).

DOR, APL, and NAP strains differed from *A. diazotrophicus* in various phenotypic characteristics, but all of these isolates were able to grow at pH 5, oxidize ethanol to acetic acid in neutral and acid conditions, and oxidize acetic acid and lactic acid to CO_2, fundamental phenotypic features for the identification of the genus *Acetobacter*.

Additionally, the family *Acetobacteraceae* can be distinguished from other α-*Proteobacteria* by RFLPs of 16S rDNA, using *Sph*I and *Nco*I restriction enzymes. The *Acetobacteraceae* species have two internal *Sph*I sites in their 16S rDNA genes, except for *A. diazotrophicus* PAl 5^T (accession number X75618) which supposedly had an extra *Sph*I site at base 485 (Sievers et al., 1994). Recently, the *A. diazotrophicus* sequence has been corrected (Teuber, 1997), and the extra *Sph*I site was shown to be non existant. With this new sequence reported there is perfect agreement with the hybridization patterns (one 16S rRNA hybridizing band of 1.3 kb) that we reported (Jiménez-Salgado et al., 1997). Genomic DNA from the DOR, NAP and APL isolates digested with *Sph*I and *Nco*I and hybridized with the same probe showed the expected 1.3- and 1.24-kb bands, respectively.

Genetic relationship analysis among N_2-fixing acetobacteria, as determined by MLEE assays, revealed that the DOR and APL isolates form two separated clusters, which diverged largely at a genetic distance of 0.950 from the *A. diazotrophicus* isolates cluster. NAP isolates fall in the *A. diazotrophicus* isolates cluster.

DOR, APL, and NAP isolates were hybridized to *A. diazotrophicus nifHDK* genes. Three hybridizing bands were observed for *A. diazotrophicus* and DOR isolates. One of this bands differed slightly in size (Table 1). NAP strains showed a more variable pattern of the *nifHDK* genes. No hybridization was observed under stringent conditions with APL strains, but at lower stringency slot DNA hybridization showed a positive signal.

RFLP analysis of *Eco*RI DNA digests from *A. diazotrophicus* and DOR and APL isolates showed distinct hybridization patterns to 16S rRNA genes, but two common hybridizing bands were observed (Table 1). *A. diazotrophicus* and NAP strains showed the same hybridization pattern.

Restriction of the PCR product of 16S rDNA of *A. diazotrophicus*, DOR and APL isolates shows differences in their corresponding pattern. *Dde*I, *Hinf*I and *Msp*I

Table 1. Genetic features of *A. diazotrophicus* and other N_2-fixing bacteria recovered from the rhizosphere of coffee plants

Type of isolate[a]	Sizes (kb)[b]		DNA-DNA homology (%)[e]
	nifHDK genes[c]	16S rDNA genes[d]	
A.d.	9.0; 2.0; 1.25	9.3; 3.6; 2.3; 1.6	100.0
DOR	9.0; 2.0; 1.20	9.7; 3.6; 1.6	14.0
NAP	7.6; 3.5; 1.20; 1.0	9.3; 3.6; 2.3; 1.6	30.0
APL	Not detected[f]	8.6; 3.9; 3.6; 1.6	13.0

A.d. = *A. diazotrophicus*.
[a]Types described in Materials and methods.
[b]Bands from total *Eco*RIDNA fingerprints.
[c]Hybridized with a *nifHDK* probe of *A. diazotrophicus*.
[d]Hybridized with a 16S rDNA probe of *E. coli*. (nucleotides 80–653).
[e]Homology (mean among isolates) to the strain PAI 5^T.
[f]Detected at low stringent conditions in slot NDA hybridization assays.

digestions of 16S rDNA of DOR isolates are different to *A. diazotrophicus* and to APL isolates. Riboprinting can differentiate APL from *A. diazotrophicus* and DOR isolates by *Sau*3AI digestion. The restriction pattern data of 16S rDNA genes of the DOR and APL strains obtained with all the enzymes tested were used for clustering analysis. APL isolates diverged 0.1 units from *A. diazotrophicus*, while DOR isolates diverged 1.8 units from the APL-*A. diazotrophicus* cluster.

The results of DNA-DNA homology experiments are shown in Table 1. DOR, APL, and NAP isolates exhibited very low DNA homology levels, ranging from 13 to 30% with strain PAI 5^T of *A. diazotrophicus*.

4. DISCUSSION

According to Swings (1992), gram-negative aerobic bacteria that oxidize ethanol to acetic acid in neutral or acid media are candidates for the family *Acetobacteraceae*. On the basis of these and other phenotypic features, as well as internal *Sph*I and *Nco*I restriction sites in 16S rDNA genes, we considered that the N_2-fixing DOR, APL, and NAP isolates belong to the family *Acetobacteraceae*. This family is divided into the genera *Gluconobacter* and *Acetobacter*, but only this last genus is capable to oxidizing ethanol, first to acetic acid and then further to CO_2 (De Ley et al., 1984; Swings, 1992), fundamental phenotypic characteristics for the identification of the genus *Acetobacter*. On this basis, DOR, APL, and NAP isolates should be regarded as belonging to the genus *Acetobacter*.

The genus *Acetobacter* includes several species, but only the species *A. diazotrophicus* is capable of fixing N_2 (Swings, 1992). Although DOR, APL, and NAP isolates were capable of fixing N_2 under microaerobic conditions, these isolates should not be assigned to the species *A. diazotrophicus* because remarkable differences were observed. Considering morphological and biochemical traits, multilocus enzyme polymorphism, 16S rDNA RFLP patterns of *Eco*RI DNA digest and riboprinting 16S rDNA, the N_2-fixing DOR and APL isolates should be considered as belonging to species of the genus *Acetobacter* other than *A. diazotrophicus*, but NAP isolates could be included in this species.

DNA-DNA hybridization levels below 70% are indicative of separate species (Stackebrandt and Goebel, 1994). On this basis the N_2-fixing DOR, APL, and NAP isolates should be regarded as new N_2-fixing species of the genus *Acetobacter*.

In this study, by using polyphasic taxonomy we demostrated that DOR, APL, and NAP isolates should be included in the genus *Acetobacter* as new diazotrophic species.

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ANEXO VI.

Manuscrito enviado "Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov., and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants".

Title:

Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov., and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants.

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Running title: New N_2-fixing acetic acid bacteria

Key words: *Gluconacetobacter johannae* sp. nov., *Gluconacetobacter azotocaptans* sp. nov., acetic acid bacteria, nitrogen fixation, coffee plants

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ABSTRACT

Diazotrophic bacteria were isolated in two different years from the rhizosphere and rhizoplane of coffee (*Coffea arabica* L.) plants cultivated in Mexico and designated as type DOR and type SAd isolates. They showed characteristics of the family *Acetobacteraceae*, having some features in common with *Gluconacetobacter* (formerly *Acetobacter*) *diazotrophicus*, the only known N_2-fixing species of acetic acid bacteria, but they differed from this species with regard to several characteristics. Type DOR isolates can be differentiated phenotypically from the type SAd isolates. Both type DOR isolates and type SAd isolates can be differentiated from *G. diazotrophicus* by growth features on culture media, by use of amino acids as nitrogen sources and by the carbon source usage. These results together with the electrophoretic mobility patterns of metabolic enzymes and amplified rDNA restriction analysis (ARDRA) suggested that the type DOR and type SAd isolates represent two new N_2-fixing species. Comparative analysis of the 16S rRNA sequences revealed that strains CFN-Cf55^T (type DOR isolate) and CFN-Ca54^T (type SAd isolate) were closer to *G. diazotrophicus* (both strains with sequence similarities of 98.3%) than to *G. liquefaciens*, to *G. sacchari* (similarities < 98%), or to any other acetobacteria. Strain CFN-Cf55^T exhibited low levels of DNA-DNA reassociation with type SAd isolates (mean homology, 42%), and strain CFN-Ca54^T exhibited a mean DNA-DNA reassociation of 39.5% with type DOR isolates. Strains CFN-Cf55^T and CFN-Ca54^T exhibited very low DNA reassociation levels, ranging from 7 to 21% with other closely related acetobacterial species. Based on these results, two new N_2-fixing species are proposed within the family *Acetobacteraceae*, *Gluconacetobacter johannae* for the type DOR isolates with strain CFN-Cf55^T as type strain and *Gluconacetobacter azotocaptans* for the type SAd isolates with strain CFN-Ca54^T as type strain.

INTRODUCTION

It is well-known that the current inventory of the bacterial species is incomplete (Dykhuizen, 1998). Therefore exists a large bacterial group that remains unknown and many species are yet to be identified.

Bacteria of the family *Acetobacteraceae* are phenotypically characterized by their ability to grow at low pH values and by their ability to oxidize ethanol to acetic acid (De Ley *et al.*, 1984a; Swings, 1992). This family has been historically divided into the genera *Acetobacter* and *Gluconobacter* (De Ley *et al.*, 1984a; Swings 1992). However, the classification of the acetic acid bacteria group has been subject to controversy. For instance, the transference of the methylotrophic species *Acetobacter methanolicus* to a new genus, *Acidomonas*, has been proposed (Urakami *et al.*, 1989) and is supported by 5S rRNA sequence data (Bulygina *et al.*, 1992), but the creation of this new genus has been criticized and was therefore not recognized (Sievers *et al.*, 1994; Swings, 1992). Similarly, the establishment of the subgenus *Gluconoacetobacter* (Yamada & Kondo, 1984) has been questioned (Swings, 1992). Recently, Yamada *et al.* (1997) proposed a division of the acetic acid bacteria into four genera, *Acetobacter*, *Gluconobacter*, *Gluconoacetobacter* and *Acidomonas*, based on the analysis of partial 16S rRNA sequences. In this proposal, only the species *A. aceti* and *A. pasteurianus* were maintained into the genus *Acetobacter* and the species *A. diazotrophicus*, *A. europaeus*, *A. hansenii*, *A. liquefaciens*, and *A. xylinus* were transferred into the genus *Gluconoacetobacter*, which has been subsequently corrected to *Gluconacetobacter* (Yamada *et al.*, 1998). In addition to the species referred to in the study of Yamada *et al.* (1997), two new species, *Acetobacter oboediens* and *A. pomorum*, have been recently described (Sokollek *et al.*, 1998). However, the species *A. oboediens* has been misnamed and should be reassigned to the genus

Gluconacetobacter (Franke *et al.*, 1999). Recently, *Gluconacetobacter sacchari*, a new species of acetic acid bacterium, has been described (Franke *et al.*, 1999).

Gluconacetobacter (formerly *Acetobacter*) *diazotrophicus*, an endophytic bacterium (Cavalcante & Döbereiner, 1988; Fuentes-Ramírez *et al.*, 1993), is the only known N_2-fixing species belonging to the acetic acid bacteria group (Gillis *et al.*, 1989) and is suggested to be a nitrogen contributor to sugar cane crops (Boddey *et al.*, 1991). For this reason therefore the search for this species has been extended to other plants. Our search for N_2-fixing bacteria associated with coffee (*Coffea arabica* L.) plants, led to the isolation of *G. diazotrophicus* (Jiménez-Salgado *et al.*, 1997). In addition, other acetic acid-producing diazotrophic bacteria from the rhizosphere of coffee plants were recovered. These diazotrophs, referred to in our previous study as type DOR, SAd, NAP, and APL isolates, shared features in common with the genus *Gluconacetobacter* but they differed from *G. diazotrophicus* with respect to morphological and biochemical traits as well as genetic and molecular features.

In this study, we present an extended taxonomic analysis of type DOR and type SAd isolates, including new isolates recovered from the rhizosphere and rhizoplane of coffee plants. We present evidence indicating that these isolates represent two new N_2-fixing species within the genus *Gluconacetobacter*. We propose the name *Gluconacetobacter johannae* for the type DOR isolates and *Gluconacetobacter azotocaptans* for the type SAd isolates.

METHODS

Isolation and reference strains. Rhizosphere soil around the root and root samples from coffee (*Coffea arabica* L.) plants var. Caturra collected in Motozintla, Chiapas State, Mexico, were treated as described previously (Jiménez-Salgado *et al.*, 1997) and inoculated into vials containing 5 ml of N-free semisolid LGI medium (Cavalcante & Döbereiner, 1988). Nitrogen-fixing type DOR and SAd isolates and strains representative of the different species of the family *Acetobacteraceae* used in this study are shown in Table 1.

Phenotypic characterization. To determine their phenotypic properties the strains were grown at 29°C in all assays. An inoculum was prepared by growing each type DOR or SAd isolate and *G. diazotrophicus* strain for 12 h in SYP medium (Caballero-Mellado & Martínez-Romero, 1994), modified by increasing the yeast extract to 0.3% (w/v). Other acetobacteria species were grown in MESMA liquid medium (Fuentes-Ramírez *et al.*, 1999). The cultures were twice centrifuged and resuspended each time in 10 mM $MgSO_4 \cdot 7H_2O$. Each culture was streaked on solid media to determine their phenotypic characteristics. Four replicates were used for each characteristic examined; growth was recorded 5 days after incubation. Colony morphology was examined on LGI (Cavalcante & Döbereiner, 1988) agar plates supplemented with 0.005% yeast extract, and on potato agar (Cavalcante & Döbereiner, 1988) containing 5, 10 or 15% cane sugar (w/v). Compounds used as sole carbon sources were tested in the presence and absence of growth factors from yeast extract (0.005%) using LGI medium supplemented with 0.1% NH_4Cl and with cane sugar replaced by 0.5% filter sterilized (pore 0.22 μm) carbon substrates: D-arabinose, L-fructose, D-galactose, D-glucose, D-lactose, maltose, mannose, melibiose, D-raffinose, L-rhamnose, sucrose, D-threalose, D-xylose, dulcitol, glycerol, D-mannitol, myo-inositol, D-

sorbitol, fumaric acid, gluconic acid, D-glucuronic acid, lactic acid, DL-malic acid, succinic acid, and starch, but acetic acid and citric acid were also tested at a concentration of 0.1%,. The following alcohols were tested at concentrations of 0.1 and 0.5% (v/v) butanol, ethanol and methanol. Sucrose was used as a positive control, while the negative control did not contain a carbon substrate. When amino acids were tested as sole nitrogen sources, the LGI medium was modified by omitting cane sugar and adding sorbitol at a final concentration of 0.5% (w/v). LGI modified medium containing NH_4Cl (0.1% w/v) was used as a positive control, while the negative control lacked a nitrogen source. The same LGI basal medium (without cane sugar) was used to test L-amino acids as carbon and nitrogen sources. LGI basal medium containing sucrose (0.5%) and NH_4Cl (0.1%), and lacking both carbon and nitrogen sources were used as positive and negative controls, respectively. Filter sterilized (pore 0.22 μm) L-amino acids were added at a final concentration of 0.1%, in both assays described above and included L-aspartic acid, L-alanine, L-cysteine, L-glutamic acid, L-glycine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, and L-trypthophan. Unless stated otherwise, LGI culture medium was adjusted with HCl to a final pH 5.5 for the above described tests and production of acid was recorded. In LGI medium, bromcresol green (0.0025%) as pH indicator was used instead of bromothymol blue. Additional tests included production of water-soluble brown pigments, oxidation of ethanol to acetic acid, and oxidation of acetate or lactate to CO_2 and H_2O on GYC medium (De Ley *et al.*, 1984b); growth at 29 and 37°C at pH 4.0, 5.0, 6.0, 7.0, 7.5, and 8.0, and tolerance to NaCl at concentrations of 0.25, 0.50, 1.0, and 1.5% (w/v) were determined in LGI liquid medium supplemented with 0.005% yeast extract.

Multilocus enzyme electrophoresis (MLEE). Cell extracts for MLEE assays were prepared as described previously (Caballero-Mellado & Martínez-Romero, 1994). Starch gel electrophoresis and the selective staining of 10 metabolic enzymes were done by methods previously described (Selander *et al.*, 1986). The following 10 metabolic enzymes activities were assayed: indophenol oxidase, lysine dehydrogenase, leucine dehydrogenase, xanthine dehydrogenase, alcohol dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, esterases and a unidentified dehydrogenase. These enzymes were analyzed under the same conditions described in a previous report (Caballero-Mellado *et al.*, 1995). Distinctive combinations of alleles for the 10 enzyme loci were designated different electrophoretic types (ETs) (Selander *et al.*, 1986). The dendrogram to illustrate the relatedness among strains was obtained from the programs ETDIV and ETCLUS from T. S. Whittam, kindly provided by B. D. Eardly (Pennsylvania State University). *G. diazotrophicus* strains (PAl 5^T, UAP 5560 and UAP-Cf 05), and type strains of *Gluconacetobacter hansenii*, *G. liquefaciens*, *Acetobacter aceti* and *A. pasteurianus* were included as references in MLEE assays to determine the relationships of the new N_2-fixing acetobacteria and well-known species of *Gluconacetobacter*, including *G. diazotrophicus*.

DNA isolation, restriction fragment length polymorphism (RFLP) of 16S rDNA genes, and DNA-DNA reassociation analysis. Cultures were grown in SYP or MESMA liquid media for 16 h and were centrifuged at 12000 g and total DNA was prepared by using a DNA/RNA isolation kit (USB, Amersham, England). For distinguishing the family *Acetobacteraceae* from other α-*Proteobacteria*, hybridization patterns of 16S ribosomal DNA (rDNA) genes were analyzed as previously described (Jiménez-Salgado *et al.*, 1997). Total DNA from the N_2-fixing acetobacteria were restricted with enzymes *Sph*I and *Nco*I, and Southern blots

hybridized with a 16S rDNA probe. In addition, DNA was digested with *Eco*RI and electrophoresed in vertical 1.0% agarose gels and total DNA digests were transferred from gels to nylon filters by the Southern procedure as previously described (Caballero-Mellado & Martínez-Romero, 1994). DNA relatedness was based on relative levels of reassociation to ^{32}P-labeled DNA by using the *redi*prime DNA labelling system (Amersham, England). The labeled DNAs, in independent experiments, were from strains CFN-Cf55^T (type DOR isolate), and CFN-Ca54^T (type SAd isolate). DNA-DNA reassociation was for 12 h at 65°C, and the nylon filters were washed once in 2 x SSC at room temperature for 10 min and once in 1 x SSC for 5 min at 65°C. Autoradiography was performed for 4 h; filter lanes were cut and radioactivity estimated with a Beckman scintillation counter. The percentage of reassociation was calculated for each strain tested in relation to the homologous control.

Amplified rDNA restriction analysis (ARDRA) and nucleotide sequence of 16S rRNA genes. The 16S rDNA genes from strains CFN-Cf55^T and CFN-Ca54^T were PCR amplified with the primers fD1 and rD1 (Weisburg *et al.*, 1991), using the proof-reading *Pwo* DNA polymerase (Boehringer-Roche). The PCR conditions were as follows: initial denaturing cycle (95°C, 3 min), 35 amplification cycles (95°C, 1 min; 55°C, 1 min; 72°C, 2 min), and then a final elongation cycle (72°C, 3 min). Approximately 400 ng of the PCR amplified 16S rRNA gene fragment (ca. 1.5 kb) were restricted with 10 U each of endonucleases *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, *Nci*I, *Rsa*I, *Sau*3AI, and *Taq*I. The lengths of the restriction fragments of the different 16S rRNA genes were determined by their electrophoretic separation in 3% agarose gels. The restriction patterns from each isolate were compared. For obtaining the nucleotide sequences, the PCR products from the strains CFN-Cf55^T and CFN-Ca54^T were initially cloned with a PCR cloning kit (Boehringer-

Roche) in the pCAPs vector, and subcloned in the pUC19 vector. The nucleotide sequences of the 16S rDNA genes were determined with an ALF automated sequencer (Pharmacia Biotech) using fluorescent primers for M13. The 16S rDNA sequences were deposited in GenBank under the accession number AF111841 for the strain CFN-Cf55^T, and AF192761 for the strain CFN-Ca54^T. The 16S rDNA sequences corresponded to positions 17 to 1524 of *E. coli* K-12 (Acc. Num. AE000460).

ARDRA and nucleotide sequence analyses. The nucleotide sequences obtained in this study were compared with different 16S rDNA sequences of acetic acid bacteria from GenBank. The multiple alignment of the sequences was performed by progressive pairwise alignments with the Wisconsin Package (version 8), based on the method of Feng & Doolitle (1987). Corrected evolutionary distances were calculated with the Wisconsin Package (version 8), using the Jukes & Cantor method (1969). Taxonomic tree files from ARDRA and DNA sequences were constructed with the neighbour-joining method, using the Clustal W Package (Thompson *et al.*, 1994). The trees were bootstrapped with Clustal W, and displayed with the TreeView program (Page, 1996). The GenBank nucleotide sequence accession numbers used were: *G. diazotrophicus* PAl5^T (X75618), *G. europaeus* DMS 6160 (Z21936), *G. hansenii* NCIB 8746 (X75620), "*A. intermedius*" TF2 (Y14694), *G. liquefaciens* IFO 12388 (X75617), *G. sacchari* SRI 1794^T (AF127407), and *G. xylinus* BPR 2001 (AJ007698) and NCIB 11664 (X75619), *Acidomonas methanolica* LMG 1668 (X77468), *Acetobacter aceti* NCIB 8621 (X74066), "*A. oboediens*" LTH 2460 (AJ001631), *A. pasteurianus* LMD 22.1 (X71863), *A. pomorum* LTH 2478 (AJ001632), *Gluconobacter asaii* IFO 3276 (X80165), *G. cerinus* IFO 3267 (X80775), *G. frateurii* IFO 3264 (X82290) and *G. oxydans* DSM 35093 (X73820). Over fifty 16S rDNA sequences (accession

numbers not shown) from acetic acid bacteria were aligned to search for descriminative *Sph*I and *Nco*I restriction enzyme sities, as previously described (Jiménez-Salgado *et al.*, 1997).

Design of specific-primers and PCR conditions. In order to obtain a rapid identification test for the type DOR and SAd isolates, specific primers were designed. The aligned 16S rDNA sequences from acetic acid bacteria showed conserved and variable regions. A region that did not show variability among the Acetobacteracea species was selected for designing an universal oligonucleotide. The variable regions were selected for designing DOR- and SAd-specific oligonucleotides. Primers were designed with the help of the software Oligo 4.0. PCR amplifications of 16S rDNA were performed both with purified DNA as well as with supernatants of cells heated at 95°C for 8 min and centrifuged for 2 min at 12000 g. PCR reactions contained the following components: $MgCl_2$ 2.5 mM, primers 20 nM, dNTPs 1 µM, *Taq* Polymerase 0.06 U per µl. PCR amplifications were performed with the primer U475 and one of the specific primers, using the following protocol: one denaturing cycle (95°C for 3) min; 32 amplifying cycles (95°C for 1 min, specific-primer annealing temperature 1 min, and 1 min at 72°C) followed by an elongation cycle (72°C for 3 min). Amplifications with the selected primers were tested both with purified DNA and with cell extracts of the strains described in Table 1, as well as *Acidomonas methanolica* ATCC 43581, *Gluconacetobacter xylinus* ATCC 700178, *Gluconacetobacter cerinus* ATCC 19441 and *Gluconobacter asaii* ATCC 43781 species.

RESULTS

Phenotypic analysis of new N_2-fixing acetobacterial species. Typical phenotypic characteristics of the N_2-fixing type DOR and type SAd isolates were compared with phenotypic features of *G. diazotrophicus*. The three N_2-fixing acetobacteria were gram-negative and motile by peritrichous flagellae (data not shown). They were catalase-positive, oxidase-negative, unable to reduce nitrates to nitrites, and capable of producing water-soluble brown pigments, however, production of these pigments was variable among isolates of *G. diazotrophicus*. In addition, these N_2-fixing bacteria were capable of oxidizing ethanol to acetic acid, and acetate as well as lactate were oxidized to CO_2 and H_2O. Growth of type DOR and type SAd isolates in N-free semisolid LGI medium resulted in the formation of a yellow surface pellicle similar to that formed by *G. diazotrophicus*, and showed acetylene reduction activity in pure culture even in presence of nitrate (10 mM) as previously described for *G. diazotrophicus* (Gillis *et al.*, 1989). However, these isolates did not exhibit typical growth of *G. diazotrophicus* on LGI agar plates. Colony morphology of type DOR isolates were shown in a previous report (Jiménez-Salgado *et al.*, 1997). DOR isolates in pure culture formed yellow-orange colonies but in contrast to *G. diazotrophicus*, they are very irregular smooth flat colonies varying in diameter from 3-5 mm after five days of growth. Type SAd colonies were similar to that of *G. diazotrophicus* with regard to their orange color but formed round, mucous, smooth convex colonies 3-5 mm in diameter, with translucent margins. On potato agar with 5, 10 or 15% cane sugar, *G. diazotrophicus* formed very characteristic dark-brown colonies after five days as described elsewhere (Cavalcante & Döbereiner, 1988), while type SAd colonies were light brown, (one isolate was reddish) but turned brownish after 10 days and produced a brownish liquid pigment. In contrast, type DOR isolates formed

only beige or very light brownish colonies even after 10 days of incubation on potato agar plates.

The three N_2-fixing acetobacteria grow and produce abundant acid on sucrose, D-glucose, and L-fructose. In addition, they were able to grow in 30% sucrose and in 30% D-glucose. Growth occurred on gluconate, but there was no acid production. The type DOR and SAd isolates differed from *G. diazotrophicus* in their ability to utilize some substrates as sole carbon sources; regardless of the presence of growth factors from yeast extract, only very few of the carbon substrates supported growth of the type DOR and type SAd isolates, as compared with the *G. diazotrophicus* species (Table 2). Growth of the type DOR and SAd isolates is slight on succinic acid. However, type DOR isolates can be differentiated from SAd isolates by their ability to grow on D-xylose, D-raffinose and 0.1% butanol. In addition, in the presence of sorbitol as carbon source type SAd isolates can be differentiated from the DOR isolates by their ability to utilize L-cysteine and L-glutamic acid as nitrogen sources, but not L-tryptophan (Table 2). Type DOR isolates produce abundant acid on D-galactose, D-xylose, and ethanol, but only slight acid production on D-raffinose, D-arabinose, maltose, D-sorbitol, glycerol, and butanol. Type SAd isolates like to DOR isolates produce abundant acid on ethanol, but in contrast they only showed slight acid production when grow on D-galactose and D-sorbitol. None of the N_2-fixing *Gluconacetobacter* species can use D-lactose, L-rhamnose, dulcitol, myo-inositol, citric acid, fumaric acid, D-glucuronic acid, DL-malic acid, starch, and methanol as sole carbon sources. The N_2-fixing acetobacteria can utilize L-alanine and L-aspartic acid as nitrogen sources when sorbitol is present as a carbon source, but only showed slight growth on L-leucine and L-lysine, and no growth on L-glycine, L-methionine or L-threonine. Single amino acids (L-alanine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glycine, L-leucine, L-lysine, L-proline,

L-methionine, L-phenylalanine, L-threonine, and L-trypthophan) cannot be used as a sole source of carbon and nitrogen by any N_2-fixing acetobacterial strain.

MLEE assays. The relationships among the N_2-fixing type DOR and type SAd isolates and reference species of *Acetobacter* and *Gluconacetobacter* are illustrated by a dendrogram based on the electrophoretic mobility of metabolic enzymes (Fig. 1). The analysis revealed that the N_2-fixing type DOR and type SAd isolates formed two unique clusters at a genetic distance of 0.560, which diverged significantly at distances of 0.920 from the *G. diazotrophicus* cluster as well as from the non N_2-fixing *Acetobacter-Gluconacetobacter* cluster.

RFLPs of 16S rDNA genes. Genomic DNA from the type DOR and type SAd isolates digested with *Sph*I and *Nco*I showed 1.3- and 1.24-kb bands, respectively, when hybridized with the 16S rDNA probe (data not shown). Such hybridization bands were previously observed in *Acetobacteraceae* (Jiménez-Salgado *et al.*, 1997).

ARDRA analysis and nucleotide sequence of 16S rRNA genes. Type DOR, type SAd and *G. diazotrophicus* isolates showed different restriction patterns for the PCR products of 16S rDNA. The patterns from *Alu*I, *Dde*I, *Msp*I, *Nci*I and *Taq*I digestions of 16S rDNA differentiated type DOR and type SAd isolates from those of *G. diazotrophicus* (data not shown). Similarly, the patterns from *Alu*I, *Dde*I, *Sau*3AI, *Taq*I and *Rsa*I digestions of 16S rDNA differentiated DOR and SAd isolates from those of *G. liquefaciens* (data not shown). Type DOR isolates can be differentiated from type SAd strains by riboprinting only with *Rsa*I digestion (Fig. 2). The 16S rDNA sequences of the strains CFN-Cf55^T and CFN-Ca54^T were aligned and compared with those of closely related bacteria present in the GeneBank database. The phylogenetic tree obtained with the 16S rDNA

sequence data of the acetic acid-producing bacteria is illustrated in Fig. 3. The genus *Gluconacetobacter* constituted a clearly cluster separated from those clusters formed by the genera *Acetobacter*, *Acidomonas* and *Gluconobacter*. Two subclusters were clearly evident within the genus *Gluconacetobacter*, one containing only non-diazotrophic species (*G. europaeus*, *G. hansenii*, and *G. xylinus*, and the misidentified species "*A. oboediens*" and "*A. intermedius*") and the other including both the non-diazotrophic *G. liquefaciens* and *G. sacchari* species, as well as *G. diazotrophicus* and the novel N_2-fixing type DOR (strain CFN-Cf55^T) and type SAd isolates (strain CFN-Ca54^T). Based on the 16S rDNA sequence analysis, the strains CFN-Cf55^T and CFN-Ca54^T (similarity of 99.31%) were closer to *G. diazotrophicus* PAl 5^T (both strains with similarities of 98.3%) than to either *G. liquefaciens* (similarities of 97.83 and 97.76%, respectively), or to *G. sacchari* (similarities of 97.83 and 97.68%, respectively).

Primer sequences. The following primers showed specific amplification of a fragment of the 16S rDNA genes of type DOR and type SAd isolates: primer U475 (5'-AATGACTGGGCGTAAAG-3', universal primer); primer L927Gj (5'-GAAATGAACATCTCTGCT-3', *G. johannae*-specific primer); primer L923Ga (5'-AATGCTCATCTCTGAACA-3', *G. azotocaptans*-specific primer). For ologonucleotide L927Gj the annealing temperature was 62°C, and 67°C for primer L923Ga. Under the conditions described those primers allowed the amplification of a ca. 400 bp fragment only from the targeted species (results not shown).

DNA relatedness. The results of DNA-DNA reassociation experiments are shown in Table 3. Type DOR isolates constituted a homogeneous group with high levels of DNA-DNA reassociation (mean = 92.75%) with reference strain CFN-Cf55^T. The strain CFN-Cf55^T exhibited relatively low level of DNA-DNA reassociation with type SAd isolates, with a mean DNA-DNA reassociation of

42%. DNAs from other related acetobacterial species, including *G. diazotrophicus*, *G. liquefaciens*, *A. aceti*, and *Gluconobacter oxydans*, exhibited very low DNA-DNA reassociation levels, ranging from 7 to 21%, with total DNA from strain CFN-Cf55^T. In addition, a homogeneous group, with relatively high levels of DNA-DNA reassociation (mean = 86.3%), was found among type SAd isolates with reference strain CFN-Ca54^T. The DNA relatedness of strain CFN-Ca54^T with type DOR isolates exhibited a mean of 39.5%, and the same strain CFN-Ca54^T exhibited less than 20% reassociation (ranging from 7 to 20%) with the other related acetobacterial species mentioned above.

DISCUSSION

It is well-known that gram-negative rod-shaped aerobic bacteria that oxidize ethanol to acetic acid in neutral or acid media are candidates for the family *Acetobacteraceae* (Swings, 1992). Additionally, the family *Acetobacteraceae* can be distinguished from other α-*Proteobacteria* by two internal *Sph*I and *Nco*I restriction sites in their 16S rDNA genes, except for *Gluconobacter oxydans* which lacks one of the *Nco*I restriction sites due to a change in the base corresponding to nucleotide 110 of *Gluconacetobacter diazotrophicus* (Caballero-Mellado *et al.*, 1999; Jiménez-Salgado *et al.*, 1997). Analysis of the 16S rDNA nucleotide sequence of the majority of acetobacterial strains reported in the genBank revealed that only few strains lack the *Nco*I restriction site (nucleotide 110). This *Nco*I restriction site is present in all the species included in the subcluster formed by the N_2-fixing type DOR and type SAd isolates, *G. diazotrophicus*, *G. liquefaciens* and *G. sacchari*. On the basis of the characteristics described above and the phenotypic features (gram-negative aerobic bacteria, oxidation of ethanol to acetic acid, oxidation of acetate and lactate to CO_2 and H_2O), it may be concluded that the N_2-fixing type DOR and type SAd isolates belong to the family *Acetobacteraceae*.

Overoxidation of ethanol, first to acetic acid and then further to CO_2 and water has been historically considered to be a fundamental phenotypic characteristic for the identification of the genus *Acetobacter* (De Ley *et al.*, 1984b; Swing, 1992), and differentiates it from *Gluconobacter* whic is not capable of overoxidizing ethanol. Members of the genus *Acidomonas*, as proposed by Yamada *et al.* (1997), can be phenotypically distinguished from closely related genera by their ability to grow on methanol. As type DOR and type SAd isolates were able to overoxidize ethanol, but did not show methylotrophic growth, these

N_2-fixing bacteria can be classified as belonging to the genus *Acetobacter* according to Swings (1992). Otherwise, they may be assigned to the genus *Gluconacetobacter* according to the proposal of Yamada *et al.* (1997).

Genetic distance at levels higher than 0.5 in MLEE analysis have been used as a criterion to suggest species limits (Musser *et al.*, 1987; Selander *et al.*, 1985). On this basis, the MLEE results strongly support the notion that the type DOR and type SAd isolates could represent two new N_2-fixing species within the family of acetic acid bacteria. The ARDRA analysis support the notion that the type DOR and type SAd isolates represent two new N_2-fixing species within the family *Acetobacteraceae*, since they can be clearly differentiated from the closely related species *G. diazotrophicus* and *G. liquefaciens*.

Althougth limitations of 16S rRNA sequencing for the differentiation of closely related species have been documented (Fox *et al.*, 1992), it is suggested that 97% is the threshold 16S rRNA similarity level for the delineation of bacterial species (Stackebrandt & Goebel, 1994). In addition, DNA-DNA reassociation levels below 70% are indicative of distinct species (Stackebrandt & Goebel, 1994). Nevertheless, some bacteria that have shown similarities over 98% in 16S rRNA sequences have been considered different species, since they had DNA-DNA reassociation levels lower than 50%. For instance, the recent descriptions of *A. pomorum* and "*Acetobacter oboediens*" (Sokollek *et al.*, 1998) and *Gluconacetobacter sacchari* (Franke *et al.*, 1999) were partially based on 16S rDNA sequence similarity higher than 97.9 or 99.0% and levels of DNA-DNA reassociation below 50%. In the present study, these considerations were consistent both with the 16S rRNA similarity levels and with the low levels of DNA relatedness exhibited among the *Gluconacetobacter* N_2-fixing cluster. In this case, the 16S rDNA sequence similarity among the type DOR and the type SAd isolates and *G. diazotrophicus* ranged from 98.3 to 99.31%, and DNA-DNA

reassociation levels were never higher than 50% in spite of the fact that the hybridization conditions were not highly stringent. Comparison of the type DOR and type SAd isolates with other species of acetic acid bacteria showed reassociation values that did not exceed 21%.

Based on data obtained from biochemical and genomic analyses, we recommend that the N_2-fixing type DOR and SAd isolates described herein should be assigned to two new species of the family *Acetobacteraceae*. We propose the name *Gluconacetobacter johannae* for the type DOR isolates with strain CFN-Cf55^T as type strain, and *Gluconacetobacter azotocaptans* for the type SAd isolates with CFN-Ca54^T as type strain.

Description of *Gluconacetobacter johannae* sp nov. *Gluconacetobacter johannae* (jo.han.'nae. L. gen. n. *johannae*, name in honor of the Brazilian microbiologist Johanna Döbereiner, who isolated the first nitrogen-fixing species of the genus *Gluconacetobacter* and discovered several other nitrogen-fixing bacteria). Cells are straight rods with rounded ends, about 1.5-1.9 µm in length by 0.5-0.6 µm in width, occurring singly, in pairs or in short chains. Motile cells by peritrichous flagella. Isolates are gram-negative, oxidase-negative and catalase-positive. The colonies on potato agar with 5, 10 or 15% cane sugare are light brown but after 10 days turned brownish and produce a brownish liquid pigment. Strains are aerobic, fix atmosferic nitrogen microaerophilically even in presence of 10 mM nitrate. Nitrates are not reduced to nitrite, but isolates grow well with ammonium. Regardless of the presence of growth factors from yeast extract, very few carbon sources support growth of the isolates. Strains grow and produce abundant acid on sucrose, D-glucose and L-fructose, and grow in 30% D-glucose or sucrose. Ethanol is oxidized to acetic acid, and acetate as well as lactate are oxidized to CO_2 and water. Isolates grow on 0.1 and 0.5% ethanol or 0.1%

butanol, but not with 0.1% methanol. Growth occurs at 29°C, but not at 37°C in LGI liquid medium at pH values from 4 to 7. Growth occurs with 0.25 and 0.5% NaCl, but not with 1.0% in LGI liquid medium. Single amino acids cannot be used as a sole source of carbon and nitrogen. Characteristics that differentiate this species from other N_2-fixing acetobacteria are shown in Table 2. This species can be differentiated from other N_2-fixing acetobacteria by ARDRA patterns, in addition to DNA-DNA reassociation data, and by means of specific primers in PCR analysis. Strain CFN-Cf55^T (= ATCC XXX; = DSM-XXX) is the type strain and was recovered from the rhizosphere of coffee plants.

Description of *Gluconacetobacter azotocaptans* sp nov. *Gluconacetobacter azotocaptans* (a.zo.to.cap'tans. N.L. n. *azotum*, nitrogen; L. part. adj: *captans*, catching). Cells are straight rods with rounded ends, about 1.6-2 μm in length by 0.5-0.6 μm in width, occurring singly, or in pairs. Motile cells by peritrichous flagella. Isolates are gram-negative, catalase-positive and oxidase-negative. Growth occurs at 29°C, but not at 37°C in LGI liquid medium at pH values from 4 to 7. The colonies on potato agar with 5, 10 or 15% cane sugare are beige or very light brownish even after 10 days. Strains are aerobic, fix atmosferic nitrogen microaerophilically even in presence of 10 mM nitrate. Nitrates are not reduced to nitrite, but isolates grow well with ammonium. Regardless of the presence of growth factors from yeast extract, very few carbon sources support growth of the isolates. Strains grow and produce abundant acid on sucrose, D-glucose and L-fructose, and grow in 30% D-glucose or sucrose. Ethanol is oxidized to acetic acid, and acetate as well as lactate are oxidized to CO_2 and water. Isolates grow on 0.1 and 0.5% ethanol, but not with 0.1% methanol. Growth occurs with 0.25 and 0.5% NaCl, but not with 1.0% in LGI liquid medium. Single amino acids cannot be used as a sole source of carbon and nitrogen. Characteristics that

differentiate this species from other N_2-fixing acetobacteria are shown in Table 2. This species can be differentiated from other N_2-fixing acetobacteria by ARDRA patterns, in addition to DNA-DNA reassociation data, and by means of specific primers in PCR analysis. Strain CFN-Ca54^T (= ATCC XXX; = DSM-XXX) is the type strain and was recovered from the rhizosphere of coffee plants.

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Table 1. Representative strains of new N_2-fixing acetobacterial species associated with coffee plants and strains of related species used in this study

Species	Type of isolated	Strain designation	Source of isolation	Reference/Source
G. johannae	DOR	CFN-Cf55^T	Rhizosphere	Jiménez-Salgado *et al.*, 1997
G. johannae	DOR	UAP-Cf57	Rhizosphere	Jiménez-Salgado *et al.*, 1997
G. johannae	DOR	CFN-Cf75	Rhizosphere	This study
G. johannae	DOR	UAP-Cf76	Rhizoplane	This study
G. azotocaptans	SAd	CFN-Ca54^T*	Rhizosphere	Jiménez-Salgado *et al.*, 1997
G. azotocaptans	SAd	UAP-Ca97	Rhizosphere	This study
G. azotocaptans	SAd	UAP-Ca99	Rhizoplane	This study
G. diazotrophicus		PAl-5^T	Sugarcane	Cavalcante & Döbereiner, 1988
G. diazotrophicus		UAP-5560	Sugarcane	Fuentes-Ramírez *et al.*, 1993
G. diazotrophicus		UAP-Cf05	Coffee	Jiménez-Salgado *et al.*, 1997
A. aceti		ATCC15973^T		American Type Culture Collection
A. pasteurianus		ATCC33445^T		American Type Culture Collection
G. hansenii		ATCC35959^T		American Type Culture Collection
G. liquefaciens		ATCC14835^T		American Type Culture Collection
Gluconobacter oxydans		ATCC19357^T		American Type Culture Collection

Abbreviations: *A*, *Acetobacter*; *G*, *Gluconacetobacter*.

*This strain was formerly designated as CFN-Cf54 (Jiménez-Salgado *et al.*, 1997).

Table 2. Comparison of the new N_2-fixing acetobacteria with *G. diazotrophicus*

Phenotypic characteristics	Type of isolate*		
	DOR (n=4)	SAd (n=3)	G.d.[√] (n=3)
Dark brown colonies on potato agar with 5, 10 or 15% sugar	No	No	Yes
Growth on the following carbon sources[&]:			
D-galactose	+	±	+
D-xylose	+	–	±/–
D-raffinose	+/±	–	+
D-arabinose	±/–	–	+
Melibiose	±	±/–	±
Maltose	+/±	±/–	±/–
Manose	–	–	±
D-sorbitol	+/±	+/±	+
Glycerol	±/–	–	+
D-mannitol	±/–	–	+
Ethanol	+	+/±	±
Butanol	±	–	–
Growth on L-amino acids in the presence of sorbitol as carbon source:			
L-cysteine	–	+	+
L-glutamic acid	–	+	+
L-proline	–	–	+
L-tryptophan	+	–	+

(+) Good growth; (±) slight growth; (–) no growth; (/) or

*DOR = *G. johannae*; SAd = *G. azotocaptans*

[√]G.d. = *G. diazotrophicus*

[&]Growth on carbon sources regardless the absence or the presence of growth factors from yeast extract

n = No. of isolates

Table 3. DNA-DNA hybridization levels between representative strains of new N_2-fixing acetobacteria species and type strains of related species

Species	Reference strain	DNA relatedness (%) with: CFN-Cf55^T	CFN-Ca54^T
G. johannae	CFN-Cf55^T	100	42
G. johannae	UAP-Cf57	97	49
G. johannae	UAP-Cf76	95	33
G. johannae	CFN-Cf75	79	34
G. azotocaptans	CFN-Ca54^T	45	100
G. azotocaptans	UAP-Ca97	50	79
G. azotocaptans	UAP-Ca99	31	80
G. diazotrophicus	PAl-5^T	19	12
G. diazotrophicus	UAP-5560	21	20
G. liquefaciens	ATCC14835^T	21	18
A. aceti	ATCC15973^T	7	8
Gluconobacter oxydans	ATCC19357^T	7	7

Abbreviations: *A*, *Acetobacter*; *G*, *Gluconacetobacter*

FIGURE LEGENDS

Fig. 1. Relationships among the N_2-fixing type DOR (*G. johannae*) and type SAd (*G. azotocaptans*) isolates and reference species of *Acetobacter* and *Gluconacetobacter* determined by multilocus enzyme electrophoresis. Strain UAP-Cf76 and the strain CFN-Cf75 corresponded to the same ET of the strain CFN-Cf55^T and UAP-Cf57, respectively. The strains UAP-Ca97 and UAP-Ca99 corresponded to the same ET of the strain CFN-Ca54^T.

Fig. 2. ARDRA profiles of the N_2-fixing type DOR (*G. johannae*) and type SAd (*G. azotocaptans*) isolates digested with endonuclease *Rsa*I. CFN-Ca54^T (lane 1), UAP-Ca97 (lane 2), 100 bp molecular weigth marker (lane 3), CFN-Cf55^T (lane 4), UAP-Cf57 (lane 5), CFN-Cf75 (lane 6), UAP-Cf76 (lane 7).

Fig. 3. Phylogenetic tree showing the relationships of the N_2-fixing type DOR (*G. johannae*) and type SAd (*G. azotocaptans*) isolates among the *Acetobacteraceae*. The tree was based on 16S rRNA gene sequences data from acetic acid bacteria and represents the most likelihood tree that was obtained by progressive alignement. The bar represents 1% estimated sequences divergency.

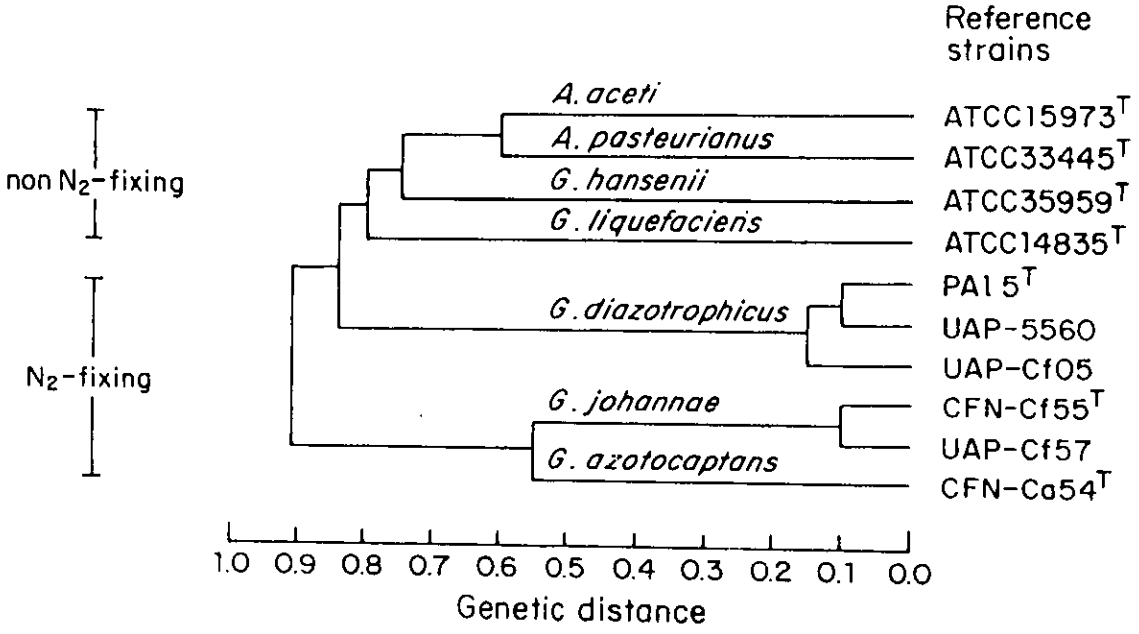
Reference strains
A. aceti
A. pasteurianus
G. hansenii
G. liquefaciens
G. diazotrophicus
G. johannae
G. azotocaptans
ATCC15973T
ATCC33445T
ATCC35959T
ATCC14835T
PA15T
UAP-5560
UAP-Cf05
CFN-Cf55T
UAP-Cf57
CFN-Co54T
non N2-fixing
N2-fixing
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
Genetic distance

Fig. 1

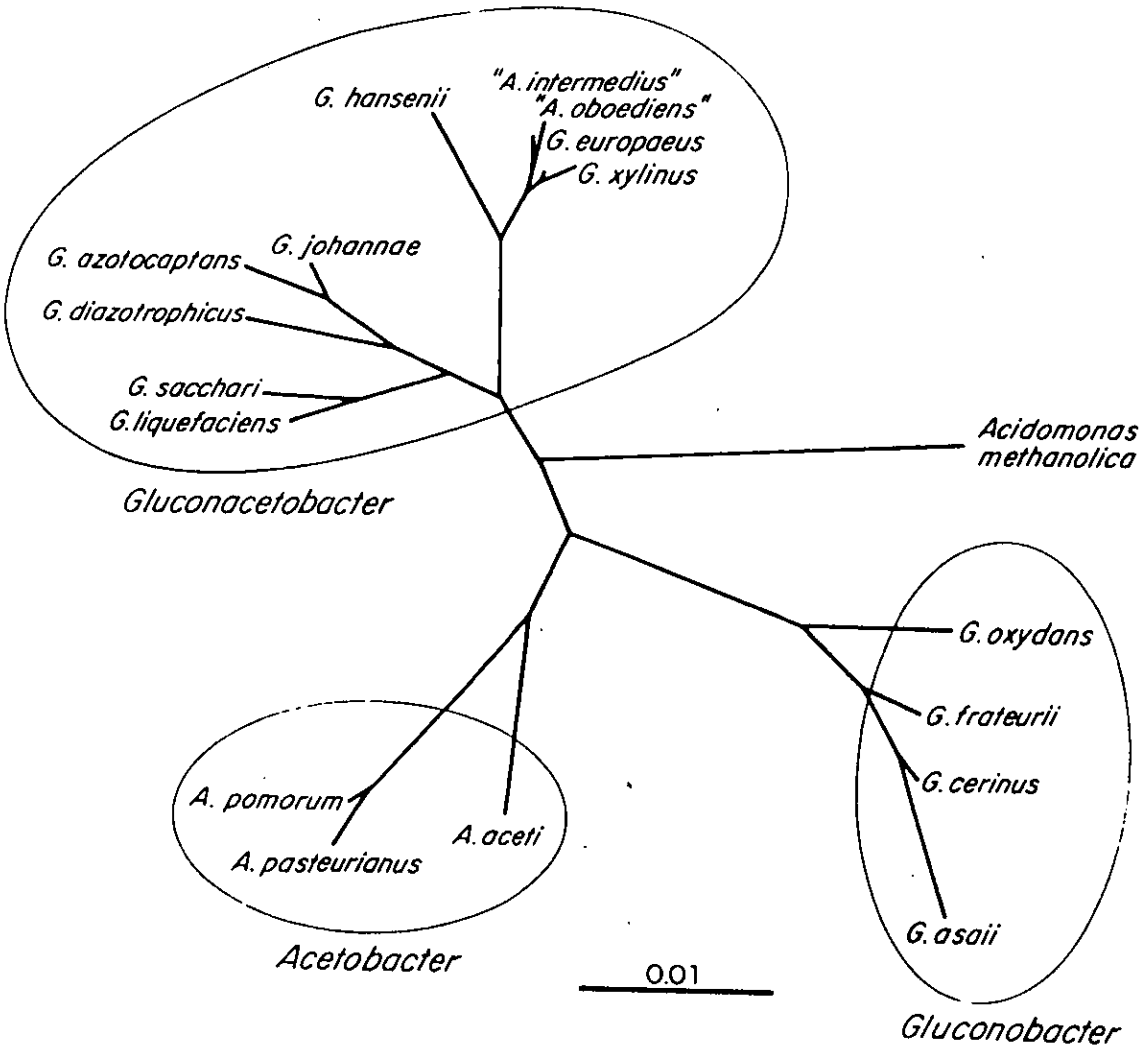
G. hansenii
"A. intermedius"
"A. oboediens"
G. europaeus
G. xylinus
G. azotocaptans
G. johannae
G. diazotrophicus
G. sacchari
G. liquefaciens
Gluconacetobacter
Acidomonas methanolica
G. oxydans
G. frateurii
G. cerinus
G. asaii
Gluconobacter
A. pomorum
A. aceti
A. pasteurianus
Acetobacter
0.01

Fig. 3