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REGULACIÓN DE LA GLUTAMINASA A DE *Rhizobium etli* Y SU PAPEL EN LA INTERACCIÓN DEL METABOLISMO DEL CARBONO Y DEL NITRÓGENO.

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El presente trabajo es la culminación de una aventura científica que se inició hacia la segunda mitad del año de 1996. En Abril de 1994 tuve el honor y la suerte de conocer al Dr. Jorge Calderón. Aún recuerdo con mucho cariño y nostalgia las horas en que discutíamos con entusiasmo el proyecto de investigación cuyos resultados ahora presento. El proyecto prácticamente se mantuvo sin cambios, a pesar de que el extraordinario maestro y buen amigo Jorge se separó de mi camino muy pronto. Las investigaciones siguieron adelante con la ayuda de la Dra. Socorro Durán, en el Departamento de Biología Molecular y Biotecnología del Instituto de Investigaciones Biomédicas de la UNAM.

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Durante el desarrollo de este trabajo recibí la distinción de una beca para estudios de Doctorado otorgada por la DGAPA de nuestra Universidad. Además, recibí el apoyo del PAEP de la UNAM.

Soy un hombre tan afortunado, que tengo muchas personas que me quieren y apoyan incondicionalmente, debo agradecer a tantas personas que son muy importantes para mí, que me limitaré a decir muchas gracias a:
Mi familia, mis amigos, mis compañeros de laboratorio, mis maestros, mis directores de tesis, mi comité tutoral, mis sinodales... a todos y cada uno mi más cálido agradecimiento.

Quiero dedicar con mucho cariño este trabajo a Enrique Huerta García (mi papá), a Jorge Calderón Jiménez (mi maestro), a Carmen Saquero Hernández (mi mamá) y a Rocío (la mujer que amo).

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RESUMEN

En *Rhizobium etli* la asimilación de amonio y la síntesis de glutamina se llevan a cabo a través de la vía de la glutamina sintetasa-glutamato sintasa (GS-GOGAT). La glutamina es degradada por diferentes enzimas, entre ellas las glutaminasas A y B, lo que origina un ciclo de síntesis y degradación simultánea de glutamina, cuyo único significado aparente es el gasto de ATP y de poder reductor. La glutaminasa A es una enzima con una alta capacidad catalítica, es indispensable para utilizar a la glutamina como fuente de carbono y participa en mantener el balance intracelular de los donadores universales de nitrógeno (glutamina y glutamato).

El ciclaje de la glutamina representa un modelo de regulación metabólica muy atractivo, por lo que nos interesó conocer los mecanismos regulatorios a nivel transcripcional y posttraduccional que operan sobre la glutaminasa A y determinar su papel en la interacción del metabolismo del carbono y del nitrógeno.

Para tales propósitos, llevamos a cabo la caracterización molecular del gen *glsA* que codifica para la glutaminasa A, su clonación en vehículos de expresión y la purificación de la enzima.

Para evaluar la regulación a nivel transcripcional de la glutaminasa A se llevaron a cabo fusiones transcripcionales de la región promotora de *glsA* y el cassette transcripcional *uidA2-aad* (del plásmido pWM5).

Por otro lado, se purificó a homogeneidad la glutaminasa A y se determinaron sus parámetros cinéticos y bioquímicos, así como la influencia de moduladores alostéricos sobre su actividad enzimática.

El gen *glsA* que complementa a la mutante LM16 (sin actividad de glutaminasa A), consta de 927 nucleótidos que codifican para una proteína de 309 aminoácidos. La secuencia de la proteína deducida presentó alta identidad con las glutaminasas A y B de *Escherichia coli* (53 y 43% respectivamente).

Los niveles de expresión de *glsA* varían dependiendo de las condiciones de crecimiento y de la fuente de carbono proporcionada. La máxima expresión de *glsA* se observa durante la fase exponencial de crecimiento, la cual disminuye durante la estacionaria, en paralelo con la actividad enzimática de glutaminasa A. La glutamina induce la expresión de *glsA*.

La glutaminasa A presenta una Km de 1.5 mM por glutamina, una Vmax de 80 µmoles de amonio min^{-1} mgP^{-1}, no se inhibe por glutamato, se activa por amonio y la carga energética de la bacteria parece no influir en su actividad. El oxaloacetato y el glioxilato la modulan positivamente, mientras que el 2-oxoglutarato y el piruvato la modulan negativamente. Las actividades enzimáticas de GS y glutaminasa A se presentan de manera simultánea durante el crecimiento bacteriano puesto que sus mecanismos de control no son recíprocos.

La sobreexpresión de la glutaminasa A aumenta la actividad de la GS y ejerce una influencia negativa en el crecimiento bacteriano, probablemente al incrementar la velocidad del ciclaje de la glutamina con su respectivo gasto de ATP y de poder reductor.

Proponemos al ciclaje como un mecanismo que permite a la bacteria direccionar el flujo metabólico hacia la utilización de fuentes alternativas de carbono (aminoácidos, glutamina en especial), cuando otras fuentes de carbono no están disponibles o son limitantes. Cuando la fuente de carbono no es limitante, el ciclaje permitiría redireccionar el flujo metabólico hacia la biosíntesis de aminoácidos u otros metabolitos nitrogenados a partir de glutamato y glutamina.

Rhizobium etli GLUTAMINASE A REGULATION AND ITS ROLE IN THE INTERACTION OF CARBON AND NITROGEN METABOLISM.

SUMMARY

In *Rhizobium etli* ammonium assimilation and glutamine biosynthesis proceeds mainly through the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. Glutamine is degraded by different enzymes, among them glutaminases A and B, which originates an ATP-consuming cycle of glutamine synthesis and simultaneous degradation.

Glutaminase A has the highest glutamine-degrading activity, playing a catabolic role in the degradation of glutamine to carbon skeletons. It also maintains the optimal intracellular balance of the nitrogen universal donors (glutamine and glutamate).

Glutamine cycling represents a very attractive model of metabolic regulation. We were interested in determining glutaminase A transcriptional and posttranslational regulation mechanisms and in determining its role in the interaction between carbon and nitrogen metabolism.

Here, we characterized the *glsA* gene, encoding glutaminase A and we expressed the gene in *E. coli* and *R. etli* strains.

To evaluate *glsA* transcriptional regulation, we constructed transcriptional fusions using the *uidA2-aad* cassette (pWM5) and the *glsA* promoter.

On the other hand, glutaminase A was purified to homogeneity by recombinant methods and its kinetic and biochemical parameters were determined, as well as the influence of allosteric modulators.

glsA gene complements LM16 mutant which lacks glutaminase A activity and predicts a 309 amino acid protein showing high similarity to glutaminases A and B from *Escherichia coli* (53 and 43% identity respectively).

The expression levels of *glsA* change depending on growth conditions and carbon source quality. The highest *glsA* expression observed during exponential growth phase decreases during stationary phase in parallel with glutaminase A activity. Glutamine induces *glsA* expression.

Glutaminase A shows a Km for glutamine of 1.5 mM and a Vmax of 80 µmol ammonium min^{-1} mgP^{-1}; it is not inhibited by glutamate, is activated by ammonium and the energetic charge of bacteria seems not to influence its activity. Oxaloacetate and glyoxilate modulate activity positively, whereas 2-oxoglutarate and pyruvate modulate it negatively. The enzymatic activities of both GS and glutaminase A are present simultaneously during bacterial growth since their control mechanisms are not reciprocal.

Glutaminase A overexpression causes an increment in GS activity and a dramatic decrease in bacterial growth, which could be due to increase in glutamine cycling and the concomitant ATP expense.

We propose glutamine cycling as a mechanism that allows bacteria to direct their metabolic flow towards the use of alternative carbon sources (amino acids, specifically glutamine), when other sources are not available or are limiting. When carbon source is not limiting, glutamine cycling would allow to redirect metabolic flow towards the biosynthesis of amino acids or other nitrogen compounds from glutamate or glutamine.

INTRODUCCIÓN

R. etli es una bacteria Gram-negativa, aerobia, en forma de bacilo. Se considera dentro del grupo de Rhizobia de crecimiento rápido. Habita el suelo en vida libre y puede fijar nitrógeno en simbiosis con la leguminosa *Phaseolus vulgaris* (frijol), lo que representa un gran interés biotecnológico para nuestro país (López y Quintero, 1987).

La glutamina es el producto final de la asimilación de amonio, tiene un papel central en el metabolismo nitrogenado en microorganismos, y es considerada junto con el glutamato los donadores universales de nitrógeno (Fig. 1). Sus grupos amino y amido son donados para una gran variedad de vías biosintéticas (Stadtman, 1973); además se ha propuesto a la glutamina como correpresor del catabolismo nitrogenado, ya que su concentración determina la utilización del nitrógeno del medio, y la velocidad de síntesis y degradación del nitrógeno celular.

En *E. coli*, la disponibilidad de amonio y de carbono se refleja en la concentración de glutamina y 2-oxoglutarato intracelular, respectivamente, lo que regula la actividad de la glutamina-sintetasa (GS) mediante una cascada de reacciones que culmina con la modificación covalente de la enzima. En resumen, las reacciones enzimáticas que llevan a la inactivación de la GS (mediante adenilación) se estimulan por glutamina y se inhiben por 2-oxoglutarato, mientras que las reacciones que reactivan a la GS (desadenilación) se inhiben por glutamina y se estimulan por 2-oxoglutarato (Halpern, 1988).

En *Saccharomyces cerevisiae*, la concentración intracelular de glutamina modula la actividad de las TOR-cinasas (TOR1 y TOR2 fosfatidilinositol-cinasas), las cuales activan el crecimiento celular en respuesta a disponibilidad de nutrientes. Dichas TOR-cinasas actúan sobre activadores transcripcionales entre los que se encuentran GLN3, RTG1 y RTG3, involucrados en la expresión de los genes que codifican para la síntesis de glutamina y enzimas del ciclo de

Krebs y del ciclo del glioxilato, respectivamente. En presencia de glutamina, GLN3 y RTG1/3 son fosforilados por TOR e inactivados, mientras que cuando la concentración de glutamina baja, se defosforilan y se translocan al núcleo, donde activan la transcripción de sus genes blanco. El control que ejerce la glutamina sobre los activadores RTG's vía TOR, sugiere una relación importante de la glutamina y el metabolismo del carbono.

Debido al papel que juega la glutamina como precursor de los compuestos nitrogenados y como modulador del catabolismo del nitrógeno, su biosíntesis y degradación debe estar altamente regulada, a fin de mantener la concentración intracelular necesaria para cubrir las necesidades biosintéticas sin tener un efecto represor en el metabolismo (Soberón & González, 1987).

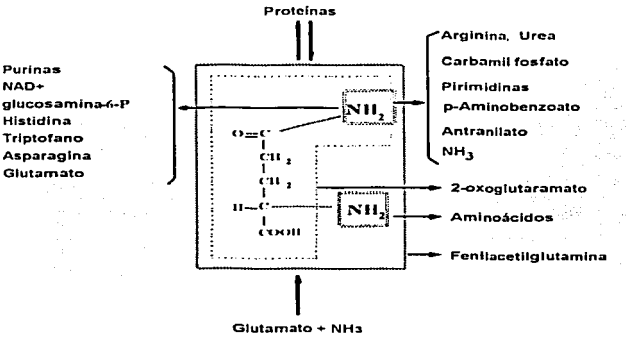


Fig. 1. La glutamina como donadora de nitrógeno.

Las glutaminasas

Las glutaminasas (L-glutamina-amidohidrolasas EC 3.5.1.2) son enzimas que catalizan la desamidación hidrolítica de la glutamina, dando como productos glutamato y amonio.

Las glutaminasas se han encontrado en varios sistemas biológicos: mamíferos, hongos, levaduras y bacterias (Calderón & Martínez, 1993; Curthoys, 1976; Durán & Calderón, 1995; Hartman & McGrath, 1973; Heini et al, 1987; Kvamme et al, 1991; Márquez, 1994; Nelson et al, 1992; Shapiro et al, 1991; Swierczynski et al, 1993).

En mamíferos, se han estudiado dos isoenzimas que presentan variación tanto en su distribución en el organismo, como en su regulación. La glutaminasa L, se encuentra únicamente en hígado de adultos y se caracteriza por una Km por glutamina elevada, es fosfato-dependiente, se activa por amonio y no se inhibe por glutamato. La glutaminasa K, se encuentra en todos los tejidos que contienen actividad de glutaminasa (riñón, cerebro, músculo, intestino delgado, tejidos fetales, tumores), tiene alta afinidad por glutamina, baja afinidad por fosfato (fosfato-independiente) y es inhibida por glutamato. Se ha propuesto que las glutaminasas en los diferentes tejidos son importantes para regular las concentraciones de glutamina, glutamato y amonio (Smith & Watford, 1990; Nelson et al, 1992; Nissim et al, 1992; Prusiner & Stadtman, 1973; Bradford & Ward, 1976; Swierczynski et al, 1993; Márquez, 1994). En hígado, la glutamina es precursor de la síntesis de glucosa y urea; en el cerebro, el amonio es tóxico aún en bajas concentraciones, por lo que la glutaminasa está altamente regulada; en riñón, el pH se regula a través de las concentraciones de amonio, en casos de acidosis, la glutaminasa se activa y regula inmediatamente el pH; en intestino, músculo esquelético y cardiaco, así como en la proliferación de células tumorales, se ha propuesto que la degradación de la glutamina por la glutaminasa es importante para utilizarla como fuente de carbono. Estudios recientes han dado luz respecto a la

organización genómica y regulación transcripcional de las glutaminasas en humano. La glutaminasa L se encuentra codificada en el cromosoma 12, mientras que la glutaminasa K se localiza en el cromosoma 2 (Pérez-Gómez et al, 2003).

En *E. coli*, se encuentran dos isoenzimas: la glutaminasa A, con un pH óptimo de 5, cuya actividad varia dependiendo de la fase de crecimiento y de la concentración de amonio, se regula por AMP cíclico y metabolitos nitrogenados; y la glutaminasa B, que es activa a pH 7, no presenta variación durante las fases de crecimiento y se regula alostéricamente por nucleótidos de adenina, cationes divalentes y ácidos carboxílicos (Halpern, 1988; Prusiner & Stadtman, 1973).

En *Bacillus licheniformis* se ha reportado actividad de glutaminasa y la existencia de dos isoenzimas: una con un pH óptimo de 7, constitutiva, y otra con un pH óptimo de 9, inducible y reprimida por glucosa (William et al, 1981). En *B. pasteurii* se ha descrito la purificación y caracterización de una glutaminasa activada por fosfato (Klein et al, 2002).

En bacterias marinas como *Micrococus luteus* K-3, *Pseudomonas fluorescens*, *Vibrio cholerae* y *V. costicola*, se ha descrito la existencia de glutaminasas tolerantes a altas concentraciones de sal, las cuales pueden ser de utilidad en la industria alimentaria, para la producción de glutamato como conservador y/o saborizante (Nandakumar et al, 1998; Renu & Chandrasekaran, 1992). *Aspergillus oryzae* tiene una glutaminasa, la cual se purificó y caracterizó. La proteína recombinante se ha utilizado en el proceso de fermentación de la salsa de soya como saborizante, pues degrada glutamina a glutamato, aumentando el sabor del producto (Koibuchi et al, 2000).

La glutaminasa también ha sido reportada en bacteroides de *R. lupini*, donde presenta una actividad muy alta, la cual aumenta en proporción al desarrollo de las plantas y a la presencia de iones Cl^- (Kretovich et al, 1985; Kretovich et al, 1982).

S. cerevisiae también tiene dos isoenzimas, una periplásmica, la glutaminasa A, y una citoplásmica, la glutaminasa B. Estas isoenzimas se distinguen por su termoestabilidad, sensibilidad a piruvato y 2-oxoglutarato y su pH óptimo (Soberón & González, 1987). Se le atribuye a la glutaminasa B la función de regular la concentración de glutamina intracelular. Cabe mencionar que a la fecha no se ha reportado la secuencia de alguna glutaminasa de *S. cerevisiae*. La búsqueda de las mismas en el GENBANK mediante homología con las secuencias de glutaminasa reportadas tampoco dio resultados satisfactorios.

Las vías de síntesis y degradación de la glutamina en *R. etli*

En *R. etli* la glutamina se sintetiza por la vía GS-GOGAT con gasto de ATP y NADPH, mientras que se degrada hasta 2-oxoglutarato y amonio a través de la vía de la transaminasa de glutamina-ω-amidasa, y a través de las glutaminasas hasta glutamato y amonio (Fig. 2) (Durán y Calderón, 1995).

La vía GS-GOGAT garantiza que las bacterias logren una eficiente asimilación de amonio aún en bajas concentraciones (Castillo et al, 2000). Esta vía parece ser necesaria para el consumo eficiente de succinato: dicho consumo es abatido cuando la actividad de GS se reduce, lo que demuestra la importante interacción existente entre el metabolismo de carbono y nitrógeno en *Rhizobium* (Encarnación et al, 1998).

R. etli tiene 3 GS's. La GSI codificada por el gen *glnA* es estructural y enzimáticamente similar a las GS's de otros procariontes, se regula por adenilación reversible, es termoestable a 50 °C y se sintetiza constitutivamente. En contraste, la GSII es diferente a las GS's de procariontes y no está sujeta a regulación por adenilación, se regula por la fuente de nitrógeno en el medio, se reprime en medios cuya fuente de nitrógeno es amonio o glutamina, y se activa

en medios con glutamato o nitrato; no se expresa en mutantes *ntrA* o *ntrC*; es termolábil y está codificada por el gen *glnII* (Bravo & Mora, 1988; Espin et al, 1994). La GSIII está codificada por el gen *glnT*, el cual no presenta similitud con el gen *glnA* ni con *glnII*.

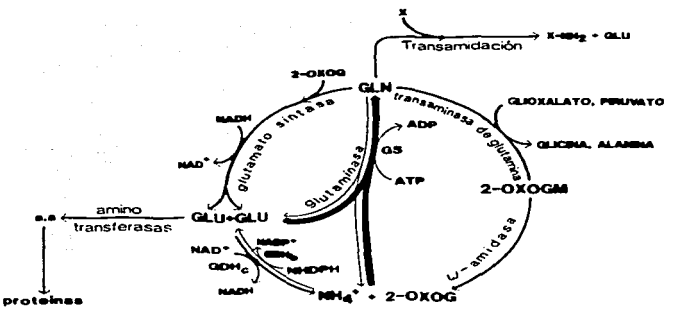
La GOGAT cataliza la transamidación reductiva de la glutamina con el 2-oxoglutarato para sintetizar dos moléculas de glutamato. Se reprime por glutamato y se inhibe por ácidos orgánicos. Puede participar en la regulación del equilibrio de la glutamina y el glutamato (Bravo & Mora, 1988). En mutantes GOGAT- de *R. etli* se evita el ciclaje de la glutamina por esta vía, lo que se traduce en un decremento del contenido de amino-nitrógeno durante la fase exponencial de crecimiento de *R. etli*, así como durante la simbiosis (Castillo et al, 2000).

La transaminasa de glutamina transfiere el grupo amino de la glutamina a un 2-oxoácido para sintetizar el correspondiente α-aminoácido y 2-oxoglutaramato. Esta reacción es reversible, pero debido a que se encuentra acoplada a una segunda reacción catalizada por la ω-amidasa, la cual utiliza como sustrato el 2-oxoglutaramato para formar 2-oxoglutarato y amonio, la vía opera en el sentido de utilización de la glutamina. Esta vía es importante para la síntesis irreversible de glicina, alanina y otros aminoácidos, así como para mantener el balance entre los mismos (Durán & Calderón, 1995).

La mutante LM16

En nuestro laboratorio se aisló y caracterizó una mutante de *R. etli* alterada en la utilización de la glutamina como fuente de carbono y nitrógeno, la LM16. Dicha mutante es incapaz de crecer en medio mínimo con glutamina como única fuente de carbono; presenta alteradas las concentraciones intracelulares de glutamina y glutamato respecto a la cepa silvestre y ha perdido la actividad de glutaminasa termolábil (actividad que representa cerca del 80 a 90% de la

actividad total de glutaminasa en la cepa silvestre), manteniendo una actividad residual y termoestable de glutaminasa. En base a la información anterior se determinó que *R. etli* tiene dos glutaminasas que se diferencian por su termoestabilidad y por su migración electroforética: una glutaminasa termoestable (B), que es constitutiva, a diferencia de la glutaminasa termolábil (A) que es regulada positivamente por glutamina y negativamente por la fuente de carbono. A diferencia de la glutaminasa A, la glutaminasa B no juega un papel importante en la utilización de glutamina como fuente de carbono, pero puede participar en mantener el balance de glutamina y glutamato (Durán & Calderón, 1995; Durán et al, 1995; Durán et al, 1996).



GLN: glutamina, **GLU:** glutamato **a.a:** aminoácido **GS:** glutamino sintetasa
NH_4^+: amonio **GDH_b:** glutamato dehidrogenasa biosintética **2-OXOG:** 2-oxoglutarato
GDH_c: glutamato dehidrogenasa catabólica **2-OXOGM:** 2-oxoglutaramato

Fig. 2. Las vías de síntesis y degradación de la glutamina en *R. etli*

La actividad de glutaminasa A de *R. etli* varía en las diferentes fases de crecimiento y con diferentes fuentes de carbono y nitrógeno, encontrándose elevada durante la fase exponencial de crecimiento, independientemente de la fuente de nitrógeno y carbono utilizada para su crecimiento, mientras que durante la fase estacionaria, la actividad de glutaminasa A baja en la mayoría de las condiciones de crecimiento, exceptuando glutamina como fuente de carbono y nitrógeno, y glutamina-succinato (Fig. 3).

La LM16 recuperó su capacidad de crecer en glutamina como única fuente de carbono y de nitrógeno mediante su complementación con el cósmido pCD24 de un banco genómico de *R. etli* construido en el vector pLAFR1 por el Dr. Jorge Calderón. Dicho cósmido contiene un fragmento de 6 Kb de DNA en el que se encuentra el gen *glsA*, que codifica para la glutaminasa A. El pCD24 se subclonó y se obtuvo el pCD2.1, el cual contiene 2 Kb de DNA donde se encuentra *glsA* y presumiblemente su región promotora, puesto que la actividad de glutaminasa de las cepas que contienen el pCD2.1 se regula de manera similar a la actividad de la cepa silvestre (Fig. 3) (Durán et al, 1996).

Fig. 3. Actividad de glutaminasa de las cepas CE3, LM16 y ambas conteniendo el plásmido pCD2.1 en diferentes medios.

El ciclaje de la glutamina

En ***E. coli***, la posible existencia de un ciclo de síntesis y degradación simultánea de glutamina, con la participación de la GS y la glutaminasa B, fue estudiado por Stanley Prusiner (1973), quien propone que la GS y la glutaminasa B tienen mecanismos regulatorios recíprocos: mientras que la GS se activa por ATP y se inhibe por AMP, la glutaminasa B se activa por AMP y se inhibe con ATP, lo que sugiere que no opera un ciclo de degradación y síntesis de glutamina (Fig. 4). Sin embargo, dicha activación e inhibición de la glutaminasa B por AMP y ATP respectivamente, solo la encuentra utilizando concentraciones muy altas de estos nucleótidos de adenina al evaluar la actividad enzimática *in vitro* (por encima de 10 mM), que difícilmente se encuentran naturalmente.

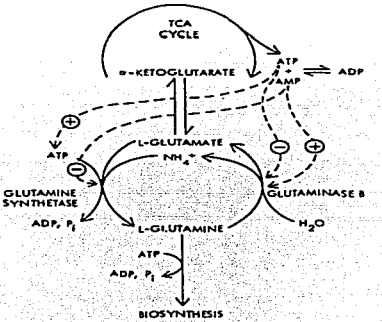


Fig. 4. Control recíproco de la GS y la glutaminasa B de *E. coli* por nucleótidos de adenina. (Tomado de Glutaminases of *Escherichia coli*: Properties, Regulation and Evolution. S. Prusiner, 1973).

En ***Neurospora crassa***, se determinó la existencia de un ciclaje de glutamina, en el que la glutamina y el 2-oxoglutarato son convertidos a dos moléculas de glutamato por la GOGAT; la glutamina también puede ser catabolizada a través de la vía de la transaminasa de glutamina-ω-amidasa hasta 2-oxoglutarato y amonio, los cuales son sustrato de la enzima glutamato deshidrogenasa (GDH), que sintetiza glutamato; por último, el glutamato producto de ambas vías puede ser convertido a glutamina por la GS. Durante esta serie de reacciones la glutamina es continuamente degradada y resintetizada, con gasto de ATP y poder reductor (Calderón & Mora, 1985; Calderón et al, 1989). El estudio de las vías de síntesis y degradación de la glutamina en *N. crassa* se complementó con estudios de marcaje isotópico de la glutamina y amonio utilizados como fuente de nitrógeno, en los que se determinó la incorporación del ^{15}N en los diferentes compuestos nitrogenados (glutamina en sus grupos amido y amino, glutamato y amonio, principalmente) (Calderón et al, 1989). Se ha sugerido que la operación de este ciclo es necesaria para mantener una utilización continua de ATP que permita un efectivo flujo de carbono, necesario para el crecimiento de este microorganismo (Hernández & Mora, 1986; Hernández et al, 1986; Mora, 1990). Es interesante mencionar que en el ciclaje de la glutamina que ocurre en *N. crassa* no se determinó la existencia de una glutaminasa. Sin embargo, con la secuenciación automática de genomas, se ha encontrado la secuencia de una probable glutaminasa para este microorganismo, la cual puede participar también en el ciclaje de la glutamina (NCBI homepage. http:// www.ncbi.nlm.nih.gov).

En ***S. cerevisiae*** se sugirió la posible existencia de un ciclo de síntesis y degradación continua de la glutamina en este microorganismo con la participación de la GS y la glutaminasa B, dicho ciclaje puede tener influencia en la relación de concentraciones de ATP/ADP intracelulares, lo que influye a su vez en el flujo glicolítico (Flores-Samaniego et al, 1993).

El ciclaje de la glutamina en *R. etli*

En *R. etli*, las glutaminasas participan en un ciclo donde la glutamina es degradada por estas enzimas y sintetizada por la GS (Fig. 5): Cuando la bacteria crece en glutamina como fuente de carbono y nitrógeno excreta altas cantidades de amonio en relación a su crecimiento en glutamina-succinato; la glutaminasa A presenta una actividad similar en ambas condiciones, mientras que la actividad de GS es muy baja cuando la bacteria crece en glutamina como fuente de carbono y nitrógeno, mientras que aumenta en glutamina-succinato. Al utilizar un inhibidor de GS, cuando la bacteria crece en glutamina-succinato, se observa que la excreción de amonio es varias veces mayor, lo que indica que el amonio liberado de la degradación de la glutamina por la glutaminasa es asimilado por la GS, lo que da lugar a la operación del ciclo de degradación y síntesis de la glutamina (Durán y Calderón, 1995). El patrón de actividad de la glutaminasa A presenta correlación con la actividad de la GS durante el crecimiento, excepto cuando la bacteria crece en glutamina como fuente de carbono y nitrógeno. En esta condición la actividad de GS se encuentra muy baja, mientras que la actividad de glutaminasa se encuentra muy elevada, por lo que el ciclaje de la glutamina opera a un nivel más bajo, permitiendo que la glutamina sea utilizada como fuente de carbono para el crecimiento.

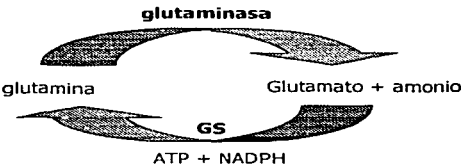


Fig. 5. El ciclaje de la glutamina en *R. etli*.

Estudios con marcaje isotópico de la glutamina [amido-^{13}N] y con amonio [^{13}N], demostraron la existencia del ciclaje de la glutamina en *R. etli* y *Sinorhizobium meliloti* (Encarnación et al, 1998). En medios con glutamina marcada y succinato, se determinó la incorporación de la marca en diferentes metabolitos nitrogenados como glutamina (en sus grupos amido y amino), glutamato, alanina y amonio, principalmente. Dicha incorporación del ^{13}N del grupo amido de la glutamina es posible solo si la degradación y resíntesis de la glutamina ocurren simultáneamente. Complementando los resultados anteriores, se determinó la distribución de la marca radioactiva de ^{13}N del amonio cuando este se proporciona como fuente de nitrógeno en medios con succinato: el amonio marcado es incorporado en glutamato, alanina y glutamina (tanto en su grupo amido como en el amino).

Se han propuesto muchas funciones para el ciclaje de la glutamina en *N. crassa*, *R. etli* y *S. meliloti:*

- ✓ Contribuir a la transaminación irreversible de la glutamina.
- ✓ Como un mecanismo de control rápido y eficiente de la concentración intracelular de la glutamina y de la velocidad de síntesis y degradación de los compuestos nitrogenados.
- ✓ Puede ser un mecanismo para tomar o liberar los esqueletos de carbono de los metabolitos nitrogenados, en función a la disponibilidad de fuentes de carbono.
- ✓ Para mantener un balance óptimo entre los compuestos nitrogenados.
- ✓ Para disipar energía, contribuir a un gasto de ATP y poder reductor, que permita una oxidación óptima de carbono en el ciclo de Krebs y con ello generar más energía.

(Calderón et al, 1989; Calderón & Mora, 1985; Mora, 1990; Encarnación et al, 1998).

Tempest y Neijssel (1987) han propuesto que los ciclos fútiles pueden funcionar como reacciones que disipan energía en condiciones en las que los procesos biosintéticos están limitados debido a una restricción de nutrientes

esenciales diferentes a la fuente de energía. La pérdida de energía puede tener sentido en tanto que permita a los microorganismos mantener sus procesos metabólicos a niveles basales y listos para incrementar su actividad cuando se requiera.

El ciclaje de la glutamina en *R. etli* es un modelo de regulación metabólica muy atractivo parece ser un punto de interacción entre el metabolismo del carbono y del nitrógeno (Poole & Allaway, 2000), por lo que nos interesó conocer los mecanismos que regulan la actividad enzimática de la glutaminasa A durante el mismo.

JUSTIFICACIÓN

La interacción del metabolismo nitrogenado con el metabolismo del carbono es evidente en la operación del ciclaje de la glutamina en *R. etli*, el cual puede ser un mecanismo que influya en los niveles energéticos de la célula, en la distribución de los compuestos nitrogenados, en derivar fuentes alternativas de carbono hacia el ciclo de Krebs, o como un mecanismo muy eficiente para permitir que la bacteria se adapte a cambios rápidos de las condiciones nutricionales, aún a expensas de un gasto importante de energía. La función de las glutaminasas durante la operación del ciclaje puede ser un punto de control, por lo que se torna necesario conocer los mecanismos regulatorios de éstas enzimas. Estos mecanismos pueden manifestarse a diferentes niveles, tanto en sus niveles de expresión genética, como a nivel de modulación de su actividad enzimática por regulación posttraduccional.

OBJETIVO

El objetivo general del presente proyecto de investigación consistió en determinar los mecanismos regulatorios que operan sobre la glutaminasa A de *R. etli* y en determinar su papel en la interacción entre el metabolismo del carbono y del nitrógeno.

OBJETIVOS PARTICULARES

- Determinar como se regula la expresión del gen *glsA* de *R. etli*.
- Determinar los mecanismos que modulan la actividad enzimática de la glutaminasa A.
- Proponer una función al ciclaje de la glutamina en base a la participación de la glutaminasa A y al conocimiento de sus mecanismos de regulación.

PLAN DE TRABAJO

El proyecto de investigación se dividió en dos vertientes para su desarrollo y cumplimiento de los objetivos planteados:

Como primera línea de investigación, la cual llamaremos la parte molecular del proyecto, nos dimos a la tarea de analizar la secuencia del gen *glsA*, que codifica para la glutaminasa A de *R. etli* y su caracterización.

La caracterización molecular del gen *glsA* y su clonación en diferentes vehículos de expresión nos permitió cumplir las siguientes metas parciales:

Sobreexpresión de la glutaminasa A para su posterior purificación, caracterización bioquímica y enzimática.

Construcción de una fusión transcripcional entre la región promotora del gen *glsA* y el cassette *uidA2-aad* (del plásmido pWM5), que contiene el gen que codifica para la β-glucuronidasa (GUS). La construcción nos permitió evaluar los niveles de expresión del gen *glsA*.

Como segunda línea de investigación, la cual llamaremos la parte fisiológica del proyecto, llevamos a cabo una evaluación de la influencia de la actividad enzimática de glutaminasa A en diferentes condiciones de crecimiento.

Llevamos a cabo la expresión controlada del gen *glsA* bajo diferentes condiciones metabólicas, a fin de evaluar el ciclaje de la glutamina *in vivo*.

Dichas evaluaciones consistieron en determinar si el ciclaje de la glutamina influye en el crecimiento de las bacterias crecidas en diferentes condiciones nutricionales con la expresión normal, sobreexpresión y falta de expresión de la glutaminasa A. Asimismo, se evaluó la velocidad de crecimiento y el rendimiento respecto a la cantidad de fuente de carbono utilizada y la cantidad de biomasa producida.

RESULTADOS

De la parte molecular del proyecto de investigación, se obtuvieron los resultados que se publicaron en los siguientes artículos:

Jorge Calderón, **Alejandro Huerta-Saquero**, Gisela Du Pont, Socorro Durán. (1999). Sequence and molecular analysis of the *R. etli glsA* gene, encoding a thermolabile glutaminase. *Biochimica et Biophysica Acta* **1444**: 451-456.

A. Huerta-Saquero, J. Calderón, R. Arreguin, A. Calderón-Flores, and S. Durán. (2001). Overexpression and Purification of *Rhizobium etli* Glutaminase A by Recombinant and Conventional Procedures. *Protein Expression and Purification* **21**: 432-437.

Los resultados obtenidos de la parte fisiológica del proyecto, se han compilado y presentado en el manuscrito que ha sido sometido a la revista *Biochimica et Biophysica Acta* para su publicación:

Huerta-Saquero, A., Calderón-Flores, A., Díaz-Villaseñor, A. and S. Durán. (2003). Regulation of *Rhizobium etli* glutaminase A. Implications to glutamine-cycling regulation. *Biochimica et Biophysica Acta*.

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Biochimica et Biophysica Acta 1444 (1999) 451–456

Short sequence-paper

Sequence and molecular analysis of the *Rhizobium etli glsA* gene, encoding a thermolabile glutaminase

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Abstract

We sequenced a 2.1 kb fragment of DNA carrying the structural *glsA* gene, which codes for the *Rhizobium etli* thermolabile glutaminase (A). The *glsA* gene complements the *R. etli* LM16 mutant that lacks glutaminase A activity, and is expressed in the heterologous host *Sinorhizobium meliloti*. The deduced amino acid sequence consists of 309 residues, with a calculated molecular mass of 33 kDa. The amino acid sequence shares 53% and 43% identity with two hypothetical glutaminases of *E. coli*; 42% identity with liver-type; 38% identity with kidney-type glutaminase; 41% and 40% identity hypothetical glutaminases of *Bacillus subtilis*; and 41% and 37% identity with two putative glutaminases of *Caenorhabditis elegans*. The *glsA* gene represents the first glutaminase gene cloned and sequenced in prokaryotes. © 1999 Published by Elsevier Science B.V. All rights reserved

Keywords: Cloning; Glutamine; Glutaminase; Thermolabile enzyme; Gene expression

Glutamine plays a key role in cellular metabolism by supplying nitrogen required for the biosynthesis of a variety of nitrogenous metabolic intermediates [1], and has been proposed as the nitrogen metabolite responsible for nitrogen catabolite repression in several microorganisms [2]. An important enzyme involved in glutamine catabolism is glutaminase, this enzyme catalyze the hydrolytic deamidation of L-glutamine resulting in the production of L-glutamate and ammonium.

Glutaminases have been detected in both prokaryotes and eukaryotes [3,4]. The presence of two glutaminases have been reported for *Escherichia coli* [5] and *Bacillus licheniformis* [6]; these can be differentiated by their regulation and pH optimum. *Saccharomyces cerevisiae* also has two glutaminases that can be distinguished by their thermostability, cellular location, pH optimum and pyruvate sensitivity [7]. In mammalian, there are also two glutaminases (liver-type and kidney-type) that can be differentiated by their size, cellular location, biochemical behavior, immunochemical cross-reactivity and regulation [4,8].

Glutaminases have been characterized at the biochemical level; however, less is known about the mechanisms involved in their regulation at the molecular level. The only published glutaminase sequences are the kidney-type [8,9] and liver-type [4] deduced from cDNA. Based on their similarity to these glutaminases, the coding sequences of the hy-

Abbreviations: *glsA* gene encoding the thermolabile glutaminase (A)

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PII: S0167-4781(99)00026-3

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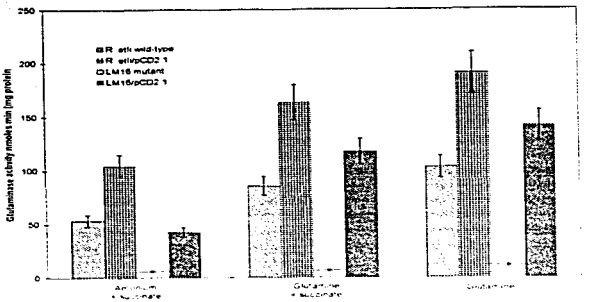

Fig. 1. Glutaminase activity after 8 h of growth under different growth conditions

pothetical *E. coli*, *Bacillus subtilis* and *Caenorhabditis elegans* glutaminases have been identified [10–12].

Rhizobium etli has two glutaminases which can be differentiated by regulation, thermostability and electrophoretic mobility. The glutaminase thermolabile (A) that is positively regulated by glutamine, and negatively regulated by ammonium and by the carbon source, and the glutaminase thermostable (B) that is constitutive [13]; these glutaminases participate in a cycle where glutamine is synthesized and degraded at the expense of ATP [14,15]. Glutaminase A plays a catabolic role in the degradation of glutamine to carbon skeletons and maintaining the balance between glutamate and glutamine, the universal nitrogen donors of the cell [13]. Here we report the nucleotide sequence of the *glsA* gene encoding glutaminase A in *R. etli*.

We previously isolated a DNA fragment of 9.1 kb (pCD24) in pLAFR1 cosmid by complementation of the *R. etli* LM16 mutant that lacks the glutaminase A activity [16]. The pCD24 cosmid was partially cut with *Hin*dIII restriction endonuclease and a subclone was isolated with a 2.1 kb DNA fragment (named pCD2.1). The pCD2.1 cosmid was introduced by conjugation into LM16 mutant, wild-type *R. etli* CFN42 and in *Sinorhizobium meliloti* 1021 strains.

Results indicate that the LM16 mutant harboring the pCD2.1 cosmid was able to grow on glutamine as the sole nitrogen and carbon source (data not shown) and glutaminase A activity was restored and regulated in the same way as in the wild-type strain

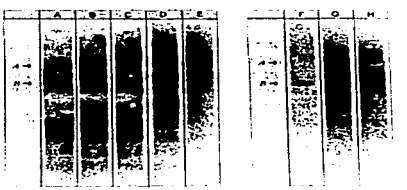

Fig. 2. In situ glutaminase activity staining in native gels. *R. etli* wild-type strain without or with heating (lanes A and F, respectively). LM16 mutant (lane B). LM16 mutant harboring the pCD2.1 cosmid (lane C). *S. meliloti* 1021 wild-type strain without or with heating (lanes D and G, respectively). *S. meliloti* harboring the pCD2.1 cosmid without or with heating (lanes E and H, respectively). The extracts were obtained from cell grown 16 h on glutamine succinate

(Fig. 1). *R. etli* harboring the pCD2.1 cosmid showed higher glutaminase A activity, which was normally regulated and *S. meliloti* harboring the pCD2.1 cosmid exhibits higher glutaminase activity (data not shown). These results led us to conclude that the 2.1 kb DNA fragment contained the complete gene encoding the *R. etli* glutaminase A.

To obtain more evidence for the presence of this gene, we performed in situ detection of glutaminase activity in polyacrylamide native gels of the LM16 mutant and *S. meliloti* wild-type strain with or without pCD2.1 cosmid and with or without heating. The LM16 mutant harboring the pCD2.1 cosmid extracts recovered the band that migrates as *R. etli* glutaminase A (Fig. 2, lane C). *S. meliloti* wild-type strain extracts showed only one band that migrated slower than *R. etli* glutaminase A (Fig. 2, lane D), and in heated extracts this band continued to be detectable but was less intense (Fig. 2, lane G). In *S. meliloti* harboring the pCD2.1 cosmid, several bands were detected: one of them migrated to the same position as *S. meliloti* wild-type glutaminase, another migrated to the same position as *R. etli* glutaminase A and three bands migrated between them (Fig. 2, lane E). These bands can be explained by the formation of hybrid oligomers of these enzymes, which have been found to be tetramers in *E. coli* [5]. In heated extracts of *S. meliloti* harboring the pCD2.1 cosmid, the band that migrated to the same position as *R. etli* glutaminase A disappeared (Fig. 2, lane H) like that in heated extracts of *R. etli* wild-type strain (Fig. 2, lane F). Finding the band in *S. meliloti* harboring the pCD2.1 cosmid that migrated as does the *R. etli* glutaminase A band, in addition to being thermolabile, indicates that we cloned the structural gene that codes for *R. etli* glutaminase A.

The nucleotide and deduced amino acid sequence of the *R. etli glsA* gene, encoding a glutaminase A is

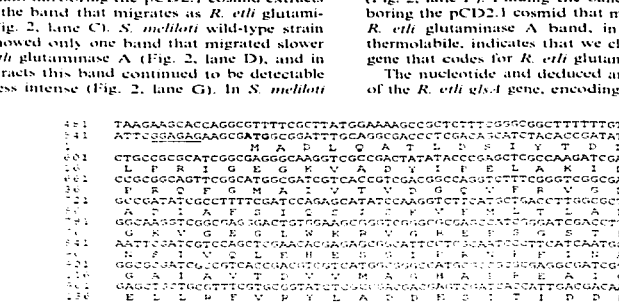

Fig. 3. The nucleotide and deduced amino acid sequences of the *R. etli glsA* gene. The translation initiation codon (ATG) is in bold type and the stop codon (TGA) is designated by an asterisk. The putative ribosome binding site is designated by underlining. The nucleotide sequence of the *glsA* gene has been deposited with GenBank under the accession no. AF057158.

```
R. etli     M....ADLQAT....L...DSIYTDILPRIGEGK.....VADYIPELAKIDPRQFGMAIV      44
E. coli 1   M...vAmdnAi....L...enIlrqvrPlIGqGK.....VADYIPaLAtvDgsrlGiAIc      45
E. coli 2   M.....Dankl....qqavDqaYTqfhslnG.Gq.....nADYIPfLAnvpgqlaavAIV      45
B. subt 1   M....vcqhn....deLealvkkakkvtdk........geVAsYIPaLAKaDkhdlsvAIy      45
B. subt 2   M...kelikehqkdinpalqlhdwveyyR...pfaangqsAnYIPaLgKvndsQlGicvl      54
Liver       fvipdfeeftghv......DrIfeDakeltGgkv......AaYIPhLAKsnPdlwGvslc     132
Kidney      fvipdfmsftshi......DelYesakkqsGgkv......ADYIPqLAKfsPdlwGvsvc     271
C. eleg 1   mvipnwvtfvdqi......rtlfneckeiregqv......AtYIPqLArqsPnlwavslc     159
C. eleg 2   miipdwerfvsdm......geIfeDvrshnegdl......AtYIPqLsrvaPdswaMsvc     196

R. etli     TVDGQVFRVGDADIAFSIQSISKVFMLTIALGKVG.EGLWHRVGRE....PSGSTFNSIV      99
E. coli 1   TVDGQlFqaGDAqerFSIQSISKVlsLvvAmrhyseEeiWqRVGkd....PSGSpFNSlV     102
E. coli 2   TcDGnVysaGDsDyrFaleSISKVctLaLALedVGpqa....VqdkigadPtGlpFNSvi     102
B. subt 1   ysnnvclsaGDvektFtlQSISKVlsLaLvLmeyGkdkvfsyVGqEpt....GdpFNSIi     101
B. subt 2   epDGtmihaGDwnvsFtmQSISKVisfiaAcmsrGipyvldFVdvEpt....GdaFNSIi     110
Liver       TVDGQrhsVGhtkIpFclQScvKpltyaisvatlGtdyvhKfV....gkePSGlryNk..     186
Kidney      TVDGQihsiGDtkvpFclQScvKplkyaiAvndlGtEyvhryV....gkePSGlrFNk..     325
C. eleg 1   TVDGQrasfGDvkhpFcvQSvSKafnyaivasdlGadvvhsyV....qqePSGrlFNe..     213
C. eleg 2   TiDGQrkmwGDAikpFclQSvSKpFtyaLvhddiGpEeLhahV....qqePSGrlFNd..     250

R. etli     QLEHESG.I.PRNPFINAGAIAVTDVVHA.GH..APREAI.GEL.....LRFVRYLADDE     149
E. coli 1   QLEmEqG.I.PRNPFINAGAlvVcD..Hlqgrlsaprqrm........LevVRqLsqvs     149
E. coli 2   aLEihqG.k.PlsPlvNAGAIAtTslinA.....Env..EqiwqriLhiqqqLAq.E       149
B. subt 1   kLEtvnp.skPlNPmINAGAlvVTslil..Gitvkeklldyllsfi........RiLtnq    150
B. subt 2   rLEinkp.gkPfNPmINAGAitiavilp..Gesayekleflysvm......etL....      155
Liver       .Lslneegiphn.PmvNAGAIvVasliklmdenkaekfdivl............qYLnkma   232
Kidney      .Lflneddkphn.PmvNAGAIvVTslikqqvnnaekfdyvm............qfLnkma   371
C. eleg 1   .icldftnkphn.PmvN-GAIviTslikkknmadridfvl............nqyrkia    259
C. eleg 2   .islonnkkphn.PlINAGAIvVaslikklpladifdfmi............hacrkfv    296

R. etli     S...ITIDDK.VARSETQT...GYRNVALAHFMPATRNLDHFVDHVL.....GVYFHQC     195
E. coli 1   d...isyDtv.VARSEfeh...saPNaAiAwlMksfqNfhHdVttVL.....qnYFHyC     196
E. coli 2   q...valsDe.VnqSEqtT...ntlNrAiAwllysagyLyedameac.....dVYtrQC     196
B. subt 1   e...ITyc.reVAe....seytamiNrAmcyyMkqYqitedlVeaVm.....dlYtkQC     197
B. subt 2   ....lnkipriheeVirsewetahRNrALAyyikettifLeacVeetL......eVYlkQC  205
Liver       gneirrifsnatf.qSEkeTqtr...NyAiq...yylkekkcfpnpVammaa.ldlYFqlC  284
Kidney      gneyvdlsnatf.qSEriodr...NfAiq...yylkekkcipeqtdmvqi.ldiYFqlC   423
C. eleg 1   gneflqinnatf.lSEraTdh...NyALs...yimkenrcipketesltdaldiYFqlC    312
C. eleg 2   gsqylqtDnsvf.lSEreTadi...NyALs...yymReinkvipkdln.lqdtldlYFqiC  348

R. etli     ALAMSCEQLARAG.LFLAAFGSNPMTGHSVVSPKRARRINALMLTCGHYDGSGDFAYHVG   254
E. coli 1   ALkMSCveLARtf.vFLAnqGkaihidepVVtPmqARqINALMaTsGmYqnaGeFAwiVG   255
E. coli 2   atllntieLAtlG.atLAAnGvNPlThkrVlqaenvpyIlAeMmmeGlYgrSGDwAYrVG   255
B. subt 1   AieMtrldLAkiG.svlAlnGihPeTGeqVisKdvARicktfMvTCGmYnaSGeFAikVG   256
B. subt 2   AmesttEdiAliG.LilAhdGyhPirheqVipkdvAklakALMLTCGmYnaSGkyAaiVG   264
Liver       svev.tcesqsvmaatLAnqGicPitGeSVlSaeavRntlsLMhsCGmYDfSGqFAfHVG   343
Kidney      siev.tcesAavmaatLAnqGicPitGerVlfPeavRntlsLMhsCGmYDfSGqFAfHVG   482
C. eleg 1   svev.tcesiavmastLAnqGvcPitnetcVdPnpcRdvlsLMysCGmYDaSGqFsfnVG   371
C. eleg 2   siet.ncdslavmaatLAnqGvNPMsGerVnnlacRdtlsLMysCGmYDwSGqFAfHVG    407

R. etli     LPGKSGVGGGIFAVAPGIASIAVWSPGLNKYGNSQLGAVALEHLAA                 300
E. coli 1   LPaKSGVGGGIvAivPhemaIAVWSPeLddaGNSlaGiavLEqLt                  300
E. coli 2   LPGKSGVGGGIlAVvPgvmaIAafSPpLdedGNSvrGqk...MvAsvakqlgynvfkg     310
B. subt 1   iPaKSGVsGGImgisPydfqiqfqPaLdekGNSiaGvkiLEim                    300
B. subt 2   vPaKSGVsGGImAlvPpsArreq..PfqsqcGigiyGp                         300
Liver       LPaKSaVsGaIllVvPnvmgmmclSPpLdKlGNShrGisfcqkL..vslfnfhnydnlrh   401
Kidney      LPaKSGVaGGIllVvPnvmgmacWSPpLdKmGNSvkGihfchdL..vslcnfhnydnlrh   540
C. eleg 1   LPaKSGVsGamivVvPnvmgiclfSPpLdslGNScrGvafckkL..vstfnfhnydclvh   429
C. eleg 2   LPaKSGVsGdmiiViPnvmgiAlySPrLdKlGNtvrGvkfaEqL..vqkynfhnydslv.   464
```


20-5

Fig. 4. Amino acid sequence alignment of *R. etli* glutaminase A with other with glutaminases sequences: *R. etli*, *R. etli* glutaminase A; *E. coli* 1 and 2, two hypothetical glutaminases from *E. coli*; *B. subt* 1 and 2, two hypothetical glutaminases from *B. subtilis*; liver, liver-type glutaminase; kidney, kidney-type glutaminase; *C. Eleg* 1 and 2, two putative glutaminases of *C. elegans*. Identical amino acids are shown in capital and boldface type. The putative active site is underlined.

shown in Fig. 3. The open reading frame was identified by comparison with published glutaminase sequences. Located 11 nucleotides upstream from the initiation codon, there is a sequence similar to a ribosome-binding site. The deduced amino acid sequence consists of 309 residues, with a calculated molecular mass of 33 kDa.

We compared the deduced amino acid sequence of the *glsA* gene with previously published glutaminase sequences. Results showed that the *R. etli* glutaminase A shared 53% and 43% identity with two hypothetical glutaminases of *E. coli*; 42% identity with liver-type; 38% identity with kidney-type glutaminase; 41% and 40% identity with hypothetical glutaminases of *B. subtilis*; and 41% and 37% identity with two putative glutaminases of *C. elegans* (data not shown). The *R. etli* glutaminase A is most similar to one of the hypothetical *E. coli* glutaminase sequences (Fig. 4, *E. coli* 1); this sequence could be the glutaminase A of *E. coli*. Both enzymes are regulated.

We examined the nine glutaminase amino acid sequences to determine conserved consensus sequences. The glutaminase sequences aligned in Fig. 4 have 54 amino acids that are conserved in the nine sequences. The data also reveal that three amino acids are conserved only in prokaryotes, while 48 amino acids are conserved in eukaryotes. We looked for a conserved consensus sequence that corresponds to the glutamine binding site (PVFGICLGHQL) previously found in the glutaminase domain of amidotransferases [9]. We did not find this sequence. This result suggests that glutaminases and amidotransferases did not evolve from a common ancestor. We also compared *R. etli* glutaminase A sequence with glutaminase asparaginase of Pseudomonas 7A [17] and we found a putative active site in the sequence SGDFAYTHVGLPGKSGVGGGI (Fig. 4).

The *glsA* gene represents the first glutaminase gene cloned and sequenced in prokaryotes. This sequence is important in order to study the regulation at molecular level of the glutaminases.

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Overexpression and Purification of *Rhizobium etli* Glutaminase A by Recombinant and Conventional Procedures

A Comparative Study of Enzymatic Properties

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Rhizobium etli **glutaminase A was purified to homogeneity by conventional procedures that included ammonium sulfate differential precipitation, ion-exchange chromatography, hydrophobic interaction chromatography, gel filtration, and dye-ligand chromatography. Alternatively, the structural *glnA* gene that codifies for glutaminase A was amplified by PCR and cloned in the expression vector pTrcHis. The recombinant protein was purified to homogeneity by affinity chromatography. This protein showed the same kinetic properties as native glutaminase A (K_m for glutamine of 1.5 mM and V_{max} of 80 μmol ammonium min^{-1} $mg\ protein^{-1}$). Physicochemical and biochemical properties of native and recombinant glutaminase were identical. The molecular mass of recombinant glutaminase A (M_r 106.8 kDa) and the molecular mass of the subunits (M_r 26.9 kDa) were estimated by mass spectrometry. These results suggest that *R. etli* glutaminase A is composed of four identical subunits. The high-level production of recombinant glutaminase A elevates the possibilities for determination of its three-dimensional structure through X-ray crystallography.** © 2001 Academic Press

Glutaminases (EC 3.5.1.2) catalyze the hydrolytic deamidation of L-glutamine resulting in the production of L-glutamate and ammonium. Glutaminases have been detected in several microorganisms: In *Escherichia coli* (1–5), *Bacillus subtilis* (6), *B. licheniformis* (7), *Saccharomyces cerevisiae* (8), *Pseudomonas aeruginosa* (9), *Micrococcus luteus* (10), *Aspergillus sojae* (11), *A. oryzae* (12), *Clostridium welchii* (13), *Rhizobium lupini* (14), *R. etli* (15–17), *Shinorhizobium meliloti* (17), *Cryptococcus albidus* (18), *Pseudomonas fluorescens*, *Vibrio cholerae*, and *V. costicola* (19). Recently, overexpression and purification of salt-tolerant glutaminase from *M. luteus* has been reported (20).

R. etli establishes nitrogen-fixing symbiosis with *Phaseolus vulgaris* (bean). *R. etli* has two glutaminases that differ in thermostability, electrophoretic mobility, and regulation (17): a thermolabile glutaminase A, which is positively regulated by glutamine and negatively regulated by the carbon source; and a constitutive and thermostable glutaminase B. Glutaminase A plays a catabolic role in the degradation of glutamine to carbon skeletons and to maintain the optimal balance between glutamine and glutamate (16, 17). *R. etli glnA* gene encoding glutaminase A has been cloned and sequenced (21).

Glutaminase A participates in an ATP-consuming cycle where glutamine is continually degraded and resynthesized (15–17, 22). We are interested in how glutaminase activity is regulated at molecular level to get insight into glutamine-cycling regulation.

In this communication we report the purification of *R. etli* glutaminase A by conventional and recombinant procedures and a comparative study of their physicochemical and enzymatic properties.

[1] In memoriam.

MATERIALS AND METHODS

Strains and Plasmids

R. etli CFN42 wild-type strain (23), *R. etli* CE3pCD2.1 (wild-type harboring the pCD2.1, pLAFR1 cosmid that contains the structural *glsA* gene) (21), and *E. coli* JM109 were used in this study. Plasmids were the pLAFR1 wide-host cosmid vector (24) and pUC18 used for cloning and sequencing of *glsA* gene; for overexpression and purification of glutaminase A, we used the pTrcHis plasmid (Invitrogen) (25).

Determination of Glutaminase Activity

Cell extracts were prepared by sonicating whole cells in ice-cold extraction buffer (0.1 M KH_2PO_4, pH 8) with a Soniprep 150 ultrasonic disintegrator; the homogenates were centrifuged and supernatants were recovered. Glutaminase activity was assayed in a 0.25-ml reaction mixture containing 0.2 ml of extraction buffer with and without 10 mM of glutamine and 0.05 ml of extract at 37°C. The reaction was stopped at different times adding 0.25 ml of 1.5% trichloroacetic acid. Ammonium released was measured as described elsewhere (15).

Determination of Enzyme Stability

Since glutaminase A activity in crude extracts is rapidly lost when submitted to dialysis, the stability of *R. etli* glutaminase A in dialysis sacs was explored with various possible stabilizers. Crude extracts of *R. etli* in the presence of possible stabilizers of the enzymatic activity were dialyzed against buffer 0.1 M KH_2PO_4, pH 8. These operations were performed at 4°C for 4 h or overnight. The feasible stabilizers included antioxidants, divalent cations, amino acids, nucleotides, and protease inhibitors. After dialysis, the enzymatic activity was measured and compared with fresh and nondialyzed extracts. Additionally, we performed glutaminase activity assays with different ionic strength of extraction and dialysis buffer (10 mM to 4 M of KH_2PO_4, pH 8).

Purification of R. etli Glutaminase A by Conventional Procedures

Batch cultures of *R. etli* CE3pCD2.1 (21) on PY-rich medium (0.5% peptone, 0.3% yeast, 7 mM $CaCl_2$) were grown overnight at 30°C with shaking at 200 rpm. Cells were washed and used as inoculum for growth on minimal medium (MM)[2] containing 10 mM glutamine and 10 mM succinate as nitrogen and carbon sources. The initial optical density (OD) of MM was adjusted to 0.05 at 540 nm. MM cultures were grown for 18 h. Cell extracts were prepared by sonicating whole cells in PG buffer (0.1 M KH_2PO_4, 5 mM glutamine, 0.5 mM DL-dithiothreitol, pH 8) with a Soniprep 150 ultrasonic disintegrator; the homogenates were centrifuged for 10 min. All operations were performed at 4°C. The supernatants were differentially precipitated with 20 and 40% of $(NH_4)_2SO_4$ (w/v). The first precipitate was discarded whereas the second precipitate was collected by centrifugation, resuspended, and dialyzed against PG buffer. The solution was chromatographed through a DEAE–cellulose column equilibrated with PG buffer. Proteins were eluted with a linear gradient (0.1–1 M KH_2PO_4) of PG buffer. The active fractions were pooled, concentrated by ultrafiltration (Amicon PM-10 membrane), and dialyzed against 1 M PG buffer. The solution was chromatographed through an octyl–agarose resin previously equilibrated with the same buffer. The enzyme was eluted with a linear gradient of PG buffer (1 M–50 mM). Active fractions were pooled, concentrated, and dialyzed against 0.2 M PG buffer. The enzyme was applied to a Sephacryl S200 gel filtration column (1.5 cm × 1 m) equilibrated with the same buffer, and the active fractions were pooled and applied to a cibacron blue column equilibrated with 0.1 M PG buffer. The column was eluted with a 0.1–1 M linear gradient of PG buffer, and the active fractions were pooled, concentrated, and stored at −70°C.

Cloning the glsA Gene into pTrcHis High-Expression Vector

We designed oligonucleotides to amplify the structural *glsA* gene that codifies for *R. etli* glutaminase A, which introduce new restriction enzyme sites at both sides of the structural gene (*Bam*HI and *Hin*dIII sites, respectively). This allows us to clone this gene into the high-expression vector pTrcHis that produces a recombinant protein with a $(His)_6$ domain at the amino end of the protein of interest.

The designed oligonucleotides were named as follows: B, 5′ cgc gga tcc ATG GCG GAT TTG CAG GCG 3′; and H, 5′ ccc aag ct TCA ATC GCC GAA AAC CGA CC 3′.

With the designed oligonucleotides and *R. etli* chromosomal DNA or pCD2.1 cosmid DNA as a template we obtained the *glsA* gene amplified by PCR. PCR conditions were as follows: initial denaturation step at 92°C for 2 min; 40 rounds of annealing (50°C for 1.5 min), primer extension (72°C for 1.5 min), and denaturation (92°C for 1 min); annealing (50°C for 2 min) and final extension at 72°C for 5 min. The fragments produced were flanked by the restriction enzyme sites *Bam*HI–*Hin*dIII. The product with the restriction sites was digested and cloned into pUC18. The new construction

[2] Abbreviations used: MM, minimal medium; OD, optical density; IPTG, isopropylthiogalactopyranoside; MALDI-TOF-MS, matrix-assisted laser desorption ionization of flight mass spectrometry; TFA, trifluoroacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

was sequenced to confirm that the sequence was identical to the *glsA* sequence previously reported (GenBank Accession No. AF057158) (15). The amplified gene was inserted into the pTreHis vector. The new construction pTrcHis/*glsA* was named pTAHS

Purification of the Recombinant Glutaminase Produced in E. coli JM109/pTAHS

The transformant JM109/pTAHS was cultured on 100 ml of LB–carbenicillin medium overnight and used as inoculum to 1 liter of the same medium to yield 0.1 OD at 540 nm. The culture was incubated at 37°C to yield 0.4 OD and then IPTG was added to a final concentration of 1 mM. The culture was incubated for 4 h at 30 C and the cells were then obtained by centrifugation and resuspended in 0.2 M KH_2PO_4, pH 8 buffer. The protein extract was prepared by sonication and diluted twice with 0.2 M KH_2PO_4 + 0.3 M NaCl + 0.1 M imidazole. The protein extract was applied to Hi-Trap prepacked column (Pharmacia) previously equilibrated with 0.1 M $NiSO_4$ and the same buffer; after several washes the recombinant enzyme was eluted with 0.2 M KH_2PO_4 + 50 mM EDTA. The recombinant glutaminase A was dialyzed against PG buffer, concentrated by ultrafiltration with an Amicon PM-10 membrane, and stored at 70 C.

Physicochemical Characterization of Glutaminases

Mass spectrometry. Nanoelectrospray mass was obtained on an Esquire ion trap mass spectrometer (Bruker-Flanzen Analytical Grmbh) as described by Jenson *et al.* (26). Protein was dissolved in 250 μl of 40:60 methanol:water, with 0.1% formic acid. Molecular weight information was analyzed using MASSMAP software.

N-terminal amino acid sequence. Tryptic in-gel digestion and matrix-assisted laser desorption ionization of flight mass spectrometry (MALDI-TOF-MS) were carried out. After SDS–PAGE, the protein band (10 μg) was excised and washed twice in 1 ml of 100 mM NH_4HCO_3. The solvent was discarded and the gel piece was washed in 500 μl of 50% (v/v) CH_3CN and 100 mM of NH_4HCO_3 for 4 h, dehydrated in 100 μl CH_3CN for 10 min, dried, and reswelled in 50 μl 100 mM NH_4HCO_3 containing 1 μg trypsin (Roche, Mannheim, Germany) for 10 min. After addition of 150 μl 100 mM NH_4HCO_3, the digestion was carried out at 37°C overnight. The supernatant was saved and the peptides were extracted with 200 μl of 50% (v/v) CH_3CN and 0.1% (v/v) trifluoroacetic acid (TFA) for 20 min. The supernatant and extracts were combined and concentrated under vacuum. The lyophilized digest was dissolved in 10% aqueous formic acid, loaded on a ZipTip (Millipore, Eschborn, Germany), washed with 0.1% TFA, and eluted in one step with 50% CH_3CN. Typically, a sample was prepared for MALDI-TOF-MS by mixing 0.5 μl of the prepared mixture and 0.5 μl matrix solution (10 mg 2,5-dihydroxybenzoic acid in 1 ml water) on the target. Postsource decay spectra were recorded on a reflectron mass spectrometer with the delayed extraction technique using a final accelerating voltage of 20 KV and a 337-nm laser (27).

Optimum pH. To study the effect of pH on isolated glutaminase activity, different buffer media were used at 100 mM final concentration: acetate (pH 5 and 5.5), Pipes (pH 6, 6.5, and 7), phosphates (pH 7.5, 8, 8.5, and 9), and carbonate buffer (pH 9.5, 10, 10.5, and 11).

The isoelectric point (p*I*) and optimum temperature were calculated by standard procedures: pI of glutaminase A was performed in PhastGel system for IEF with separation range p*I* 3 to 9 and pharmalyte carrier ampholytes (Pharmacia). Optimum temperature was calculated by performing glutaminase assays from 20 to 60°C with intervals of 5°C in 0.1 M KH_2PO_4, pH 8, with 10 mM glutamine as a substrate.

RESULTS AND DISCUSSION

Stability of R. etli Glutaminase A

Glutaminase A is an unstable enzyme which loses 20% of its activity if maintained at 4°C for 12 h, up to 70% during dialysis of crude extracts in 0.1 M KH_2PO_4 buffer, pH 8, and over 90% when dialysis is performed in water (not shown). Since the rapid inactivation is rather disadvantageous for purification, this point was investigated. Dithiothreitol (DTT) was tested as antioxidant agent, EDTA and PMSF as protease inhibitors, divalent cations as possible cofactors, and the amino acids glutamine and glutamate (substrate and product of glutaminase activity) and the nucleotides ATP and ADP as possible allosteric effectors. Additionally, the ionic strength of the dialysis buffer was modified to preserve glutaminase activity. As shown in Table 1, DTT preserved enzymatic activity of glutaminase A in about 60% compared to fresh extract, whereas glutamine 5 mM preserved the enzymatic activity by over 80%. Addition of 5 mM glutamine and 0.5 mM DTT to the dialysis buffer maintained 98% of glutaminase activity, so it was decided to add these stabilizers to all the buffers used for purification steps (PG buffer). The protective effect of DTT suggests that there are important residues susceptible of oxidation in the glutaminase structure. Glutamate and ADP showed stabilizing behavior of glutaminase activity, which preserved it in 69 and 77%, respectively. Since glutamate is the product of deamidation of glutamine by glutaminase, its protective effect on activity—as well as that of glutamine—could be explained by the interaction of the amino acids with the active site of the enzyme, thus preserving its

TABLE 1

Possible Stabilizers of Dialyzed Glutaminase A

Stabilizer	% of activity
Fresh extract	100
Without stabilizer, without dialysis	80
Without stabilizer, dialyzed	28
0.5 mM DTT	61
10 mM $CaCl_2$	38
10 mM $MgCl_2$	34
10 mM $MnCl_2$	31
1 mM Glutamine	75
5 mM Glutamine	81
1 mM Glutamate	69
0.1 mM ADP	77
10 mM EDTA	33
1 mM PMSF	22
5 mM Glutamine + 0.5 mM DTT	98

Note. Percentages with respect to total activity of fresh extract as a control. Determinations were made at least three times. Representative results are shown.

active form. The ease of ADP suggests that this nucleotide could be an allosteric effector of glutaminase A as in glutaminase B from *E. coli* (5).

Recombinant glutaminase was highly stable in PG buffer and in a broad ionic strength of the phosphate buffer (10 mM to 2 M) (not shown).

Purification of R. etli Glutaminase A by Conventional Methods

The purification steps of *R. etli* glutaminase A are summarized in Table 2 and shown in Fig. 1. Differential precipitation of cell-free extract with ammonium sulfate together with DEAE–cellulose clarify the extract to perform hydrophobic interaction chromatography in octyl–agarose, in which good purification was obtained without loss of yield. The relative molecular weight of

TABLE 2

Purification Steps of *R. etli* Native and Recombinant Glutaminase A

Step	Total protein (mg)[c]	Total activity (U)[a]	Specific activity (U/mg)[c]	Fold	Yield (%)
Cell-free extract[c]	250	75	0.3	1	100
$(NH_4)_2SO_4$	76.3	72.2	0.95	3	96
DEAE–cellulose	11	20.5	1.86	6	27
Octyl–agarose	2.6	20	7.7	26	26
Sepharyl S200	0.35	7.7	22	73	10
Cibacron blue	0.01	0.7	70	233	0.9
Cell-free extract[b]	250	850	3.4	1	100
Hi-Trap–Ni	10.3	752	73	21	88

[a] U, μmol amonnium min^{-1}.
[b] From *R. etli* CE3pCD2.1.
[c] From *E. coli* JM109/pTAHS.

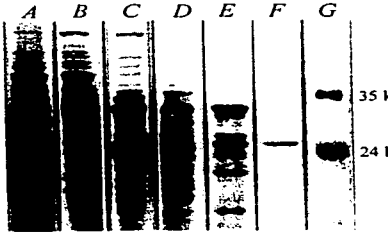

FIG. 1. SDS–PAGE of purification steps of *R. etli* glutaminase A. Protein loaded to each lane is shown in parentheses. (A) Cell-free extract (100 μg). (B) $(NH_4)_2SO_4$ precipitation (100 μg). (C) DEAE–cellulose (75 μg). (D) Octyl–agarose (75 μg). (E) Sepharyl S200 (50 μg). (F) Cibacron blue (10 μg). (G) MW markers.

native glutaminase A was calculated by gel filtration in Sepharyl S200 utilizing as standards (MW × 1000): cytochrome c (12.4), carbonic anhydrase (29), albumin (66), alcohol dehydrogenase (150), and β-amylase (200) and blue dextran (MW 2,000,000) to calculate void volume (V_o). A calibration curve was obtained by plotting the logarithms of the known molecular weights of protein standards versus their respective (V_e/V_o) values, where V_e corresponds to volume elution of each standard. Native glutaminase A molecular weight was calculated in 100,000 by this method. Dye-ligand chromatography of glutaminase A in cibacron blue was performed by the possibility of interaction of this enzyme with nucleotides, since cibacron blue has structural homology to the NAD molecule. As a result of the interaction of glutaminase A with cibacron blue, we obtained the enzyme purified near to homogeneity, as shown in Fig. 1. SDS–PAGE of glutaminase A reveals a single band of glutaminase monomers of 25.5 kDa, which suggests that glutaminase A is composed of four identical subunits.

Purification of His-Glutaminase A Produced in E. coli JM109/pTAHS

As a result of overexpression of recombinant glutaminase A we obtained more than 10 mg of soluble protein per liter of culture. Recombinant glutaminase A contains $(His)_6$ domain included in a tag fused at the amino termini. Affinity purification of recombinant glutaminase A was performed under high ionic strength conditions that avoided unspecific binding of contaminant proteins to Hi-Trap resin and also contributed to the

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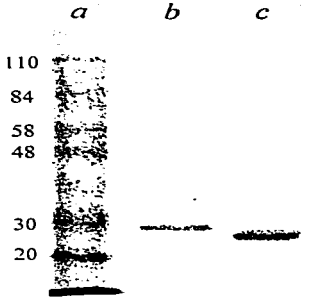

FIG. 2. SDS-PAGE of recombinant glutaminase A. 10 μg of protein was loaded to each lane. (a) MW markers. (b) Recombinant glutaminase A. (c) Native glutaminase A.

stability of the enzyme. One-step purification allowed us to recover over 90% of His-glutaminase A in active form purified to homogeneity (Fig. 2).

Kinetic Parameters of Glutaminases

Glutaminase activity was performed with purified glutaminases in 0.1 M KH_2PO_4 buffer, pH 8, at 37°C with glutamine at different concentrations (0.01 to 40 mM). Glutaminase activity maintained by recombinant glutaminase A suggests that folding of this heterologous protein in *E. coli* was successfully done and the structure of its active site remains without changes.

Results of the kinetic analysis of native and recombinant glutaminase A are summarized in Table 3. Native and recombinant glutaminase A showed the same kinetic behavior (K_m for glutamine of 1.5 mM and V_{max} of 80 μmol ammonium min^{-1} mg $protein^{-1}$). The His-tag at the amino termini of recombinant glutaminase A slightly altered cooperativity (Hill coefficient of 1.8), but catalytic properties remained unchanged.

TABLE 3

Kinetic Parameters of *R. etli* Glutaminase A

	K_m, mM[a]	V_{max}[a]	Hill
Native glutaminase A	1.5	80	2
Recombinant glutaminase A	1.5	80	1.8

[a] V_{max} = μmol ammonium min^{-1} mg $protein^{-1}$.

Physicochemical Characterization of Glutaminases

Physicochemical parameters of native and recombinant glutaminase A remained almost the same and are summarized in Table 4.

Mass spectrometry. Mass spectrometric determinations yielded a molecular mass of 106,813 Da for recombinant glutaminase A as a tetramer whereas each monomer yielded a molecular mass of 26,921 Da.

N-terminal amino acid sequence. The N-terminal amino acid sequence of the native glutaminase was determined to be MADLQATLDS, identical to that deduced from the previously reported nucleotide sequence (21).

pH profile. Different buffer media were used to study the effect of pH on glutaminase activity from native and recombinant proteins (see Material and Methods). Glutaminase activity shows a broad pH profile (more than 50% of activity is maintained between pH 6 and 10) with a maximum peak of activity at pH 8.3–8.5 (data not shown).

p I. Isoelectric focusing of native and recombinant glutaminase A was performed in Phast system gels (Pharmacia) using broad pI range markers (3.5 to 8.65). Calculated p*I* of native glutaminase A was 6.3 whereas p*I* of recombinant glutaminase A was 6.1. This slight difference of p*I* between native and recombinant glutaminase A is due to the His-tag at amino end of recombinant protein.

Optimum temperature. Glutaminase A activity of both native and recombinant proteins was determined at different temperatures. More than 50% of glutaminase activity is retained within a broad range of temperatures (from 30 to 60°C) with a major activity peak at 45°C (not shown). However, thermolability of native and recombinant glutaminase A remained as previously reported (17).

In this communication we report *R. etli* glutaminase

TABLE 4

Physicochemical Properties of Native and Recombinant Glutaminase A

MW of native protein[a]	100,000
MW of recombinant protein[a]	106,813
MW of monomers (native)[b]	25,500
MW of monomers (recombinant)[b]	26,921
pH optimum	8.3
p*I* of native protein	6.3
p*I* of recombinant protein	6.1
Temp. optimum (°C)	45

[a] Estimated by gel filtration.
[b] Estimated by mass spectrometry.
[c] Estimated by SDS-PAGE.

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A purification by conventional and recombinant procedures. The structural *glsA* gene that codifies for glutaminase A was amplified by PCR and cloned in the expression vector pTrcHis. The recombinant protein showed the same kinetic properties as native glutaminase. Physicochemical and biochemical properties of native and recombinant glutaminase were the same. The heterologous expression of recombinant glutaminase A with high-level production presented here will contribute to the future elucidation of the function–structure relationship of glutaminase through X-ray crystallographic analysis. Additionally, we have the possibility to determining glutaminase activity regulation at molecular level to get insight into glutamine-cycling regulation in *R. etli*.

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CONSIDERACIONES FINALES

Este trabajo se originó a partir de la determinación de la existencia de isoformas de glutaminasa mediante evidencias bioquímicas y fisiológicas y de la importancia de las mismas para el metabolismo nitrogenado de *R. etli*. La necesidad de conocer los mecanismos de regulación que operan sobre la expresión del gen *glsA*, así como de los que modulan la actividad enzimática de la glutaminasa A, tiene su origen en la ocurrencia del ciclaje de la glutamina. La secuenciación del gen *glsA* de *R. etli* se constituyó en el primer reporte de una secuencia de glutaminasa de procariontes. La clonación de dicho gen en diferentes vehículos nos permitió evaluar su regulación transcripcional, así como la purificación de la enzima para su posterior caracterización. El conocimiento de los mecanismos regulatorios que operan sobre la glutaminasa A, nos permitieron proponer una función al ciclaje de la glutamina en *R. etli*. Los resultados obtenidos, se discuten a continuación.

Estudios previos han demostrado la interacción entre el metabolismo de carbono y la síntesis de glutamina en *R. etli*: en medios con glutamina-succinato, la síntesis de glutamina favorece la utilización de succinato; mientras que en medios con glutamina como única fuente de carbono –donde ésta se encuentra en exceso-, su síntesis se restringe y es utilizada como fuente de energía gracias a la actividad de la glutaminasa A (Durán & Caldelrón, 1995; Encarnación et al, 1998). Dicha actividad varía en diferentes condiciones de cultivo, encontrándose más elevada en presencia de glutamina y durante la fase exponencial de crecimiento. La expresión de *glsA* se induce por la presencia de glutamina en el medio y varía en las diferentes fases de crecimiento en correlación directa con la actividad enzimática. Es importante mencionar que la expresión de *glsA* se presenta de manera importante en todas las condiciones de crecimiento probadas, lo que sugiere que la glutaminasa A es una enzima clave en el metabolismo de nitrógeno de la bacteria. La máxima inducción de *glsA* se observó en glutamina como única fuente de carbono y

nitrógeno, condición en la que la síntesis de glutamina se encuentra inhibida, por lo que el ciclaje se reduce, permitiendo la utilización de los esqueletos de carbono de la misma, a la vez que se secreta al medio de cultivo el amonio excedente. En glutamina-succinato, la expresión de *glsA* así como la actividad enzimática de glutaminasa A también es elevada, lo que permite utilizar a la glutamina como fuente de carbono, aunque se ha demostrado que *R. etli* oxida succinato preferencialmente que glutamina (Encarnación et al., 1998). En esta condición de crecimiento, la GSII se induce, por lo que se propone al ciclaje de la glutamina como una reacción de disipación de energía (Encarnación et al., 1998; Tempest, 1978; Mora, 1990). En amonio-succinato, la expresión de *glsA* y la actividad de glutaminasa A bajan respecto a las condiciones anteriores; a pesar de ello, la síntesis y degradación de la glutamina ocurre simultáneamente, como lo demuestra Encarnación et al. (1998). La presencia de glutaminasa A en esta condición sugiere que la síntesis de glutamina induce la expresión de *glsA*. El ciclaje en esta condición no tiene un significado aparente más que el gasto inútil de energía; a menos que existan mecanismos regulatorios sobre la glutaminasa A que mantengan su actividad enzimática en niveles basales o bien que exista un control antagónico entre las actividades de GS y glutaminasa A, como el propuesto en *E. coli* por Prusiner (1973).

Para evaluar dicha posibilidad, se llevó a cabo la purificación a homogeneidad de la glutaminasa A y su caracterización bioquímica y enzimática. La glutaminasa A recombinante presenta las mismas características cinéticas que la glutaminasa A nativa purificada por métodos convencionales (Huerta-Saquero et al, 2001). La glutaminasa A no es inhibida por glutamato, se activa por amonio, el ATP, ADP y AMP parecen no influir en sus niveles de actividad. El oxaloacetato y el glioxilato la modulan positivamente, mientras que el 2-oxoglutarato y el piruvato la modulan negativamente. Las actividades enzimáticas de GS y glutaminasa A se presentan de manera simultánea durante el crecimiento bacteriano y sus mecanismos de control no son recíprocos.

La inhibición por 2-oxoglutarato y piruvato sugiere una estrecha relación entre el metabolismo del carbono y la utilización de glutamina, como el propuesto por Halpern en *E. coli*, donde la síntesis de glutamina por la GS es controlada por la concentración intracelular de glutamina y 2-oxoglutarato (1988). Cuando aumenta la cantidad de carbono disponible, aumenta la concentración de piruvato y 2-oxoglutarato, lo que inhibe la actividad de la glutaminasa A, permitiendo que la glutamina sea utilizada por una vía alternativa (por ejemplo la vía de la transaminasa de glutamina-ω-amidasa), utilizando 2-oxoácidos para la síntesis de aminoácidos, como en *S. cerevisiae* (Soberón & González, 1987). La modulación positiva de la glutaminasa A por oxaloacetato y glioxilato, sugiere también una relación estrecha entre la disponibilidad de fuentes de carbono y la actividad de la glutaminasa A: El oxaloacetato es un intermediario del ciclo de Krebs que puede provenir de la vía anaplerótica de utilización de asparagina o aspartato como fuentes alternativas de carbono cuando otras fuentes son limitantes; mientras que el glioxilato es producto de la vía que lleva su nombre, la cual también se activa en condiciones limitantes de carbono. En corto, la actividad de glutaminasa A puede modularse hacia la alta cuando se requiere proveer de carbono al ciclo de Krebs, permitiendo la utilización de glutamina como fuente de carbono. Relacionado con lo anterior, en *S. cerevisiae*, se ha descrito la regulación de activadores transcripcionales de la vía TOR en respuesta a los niveles intracelulares de glutamina. Entre dichos activadores, se reconocen RTG1 y RTG3 que participan en la expresión de genes que codifican para enzimas del ciclo de Krebs y del ciclo del glioxilato (Crespo et al, 2002). La activación de la glutaminasa A por amonio permite a la bacteria crecer en glutamina como única fuente de carbono y nitrógeno, condición en la cual se excretan altas cantidades de amonio.

Por otro lado, al evaluar el rendimiento del crecimiento de la cepa silvestre y la cepa LM16 (*glsA*$^{-}$), en amonio-succinato, se observó que la LM16 presenta un rendimiento 7% mayor respecto al encontrado para la silvestre, lo

que sugiere que el ciclaje de la glutamina ejerce un gasto importante de energía para la bacteria. Al manipular el ciclaje de la glutamina sobreexpresando la actividad de la glutaminasa A bajo el control de un promotor inducible, superamos los mecanismos regulatorios de la enzima a nivel transcripcional y alostérico, lo que nos permitió amplificar el costo energético del ciclaje. Un incremento en la expresión de la glutaminasa A provocó un aumento en la actividad de GS y un decremento dramático en el crecimiento de la bacteria.

R. etli es una bacteria que habita en el suelo y que puede establecer una relación simbiótica con *P. vulgaris*. En vida libre, las condiciones nutrimentales pueden variar de manera importante, por lo que las bacterias requieren mantener una plasticidad metabólica que les permita contender con dichas condiciones cambiantes, adecuándose lo más rápido posible a las nuevas condiciones metabólicas. En este sentido, proponemos al ciclaje de la glutamina como un mecanismo que permite a la bacteria direccionar el flujo metabólico hacia la utilización de fuentes alternativas de carbono (aminoácidos, glutamina en especial) cuando otras fuentes de carbono no están disponibles o son limitantes; o bien, cuando la fuente de carbono no es limitante, redireccionar el flujo metabólico hacia la biosíntesis de aminoácidos u otros metabolitos nitrogenados a partir de glutamato y glutamina: si hay disponibilidad de fuentes de carbono, aumenta el piruvato y el 2-oxoglutarato, lo que inhibe la degradación de la glutamina por la glutaminasa A, lo que permite que ésta sea utilizada para la biosíntesis de otros aminoácidos u otros compuestos nitrogenados; si la fuente de carbono se torna limitante, la glutamina puede ser utilizada como fuente de carbono, para soportar el crecimiento bacteriano.

El ciclaje de la glutamina provoca un gasto de energía que, en condiciones nutricionales óptimas -como las proporcionadas en laboratorio- efectivamente no tiene un significado fisiológico importante, el gasto es inútil; sin embargo, parece haber sido seleccionado naturalmente por permitir a la bacteria una adecuación a los cambios en las condiciones nutricionales, aún a expensas del gasto energético que representa.

La glutaminasa A en la simbiosis

Las glutaminasas operan en bacteroides de *R. etli*, lo que sugiere que sus actividades pueden ser importantes en el proceso de simbiosis y fijación de nitrógeno (Durán & Calderón, 1995; Durán et al, 1995; Durán et al, 1996). En experimentos de simbiosis entre la mutante LM16 *y P. vulgaris*, se ha encontrado que los bacteroides acumulan glutamina y su poza de glutamato se presenta baja en relación a bacteroides de la cepa silvestre, lo que nos indica que la glutaminasa está operando durante el proceso de la simbiosis y la fijación biológica de nitrógeno (Durán & Calderón, 1995; Durán et al, 1995). La actividad específica de glutaminasa encontrada en nódulos es tan alta como cuando la bacteria crece en glutamina como única fuente de carbono y nitrógeno. Con el fin de comprobar que la actividad de glutaminasa encontrada en bacteroides corresponde a la glutaminasa A de *R. etli*, llevamos a cabo experimentos de infección de *P. vulgaris* con las cepas CE3*glsA*GUS, LM16*glsA*GUS y sus respectivos controles con las construcciones antisentido. Como resultado obtuvimos una alta inducción de la expresión de *glsA* en bacteroides de la cepa silvestre, que se incrementó de manera importante en bacteroides de la mutante LM16, lo que demuestra que la expresión de *glsA* se induce durante la fijación biológica de nitrógeno (Fig. 6). Sin embargo, la mutante LM16 nodula y fija nitrógeno atmosférico de manera similar a la cepa silvestre. El fenotipo simbiótico de la LM16 sugiere que la actividad de la glutaminasa A no es relevante para la nodulación y la fijación de nitrógeno, pero cabe la posibilidad que la actividad de la glutaminasa B compense –aún de manera parcial- la ausencia de la glutaminasa A durante la simbiosis.

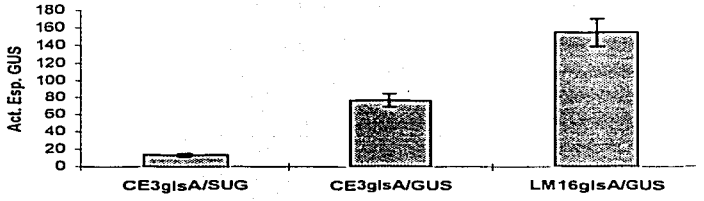

Fig. 6. Expresión de *glsA* (GUS) en nódulos

Se obtuvieron los nódulos de 5 plantas después de 30 días de ser inoculadas con las respectivas cepas. Se muestran los errores estándar

La actividad específica se calculó respecto a la proteína total del nódulo.

El catabolismo de aminoácidos ha sido ampliamente estudiado como un punto de interacción entre el metabolismo del carbono y del nitrógeno durante el intercambio simbiótico bacteroide-leguminosa. Kahn y colaboradores han propuesto que la fuente de carbono que le proporciona la planta a la bacteria durante la simbiosis puede incluir a los aminoácidos (Kahn et al, 1985); Rastogi y Watson, reportaron que mutantes de *R. meliloti* incapaces de crecer en aspartato, no fijan nitrógeno debido a la deficiente actividad de una aspartato aminotransferasa, lo que indica que el metabolismo del aspartato es esencial en el nódulo (Rastogi & Watson, 1991); En bacteroides de *Bradyrhizobium japonicum*, se ha encontrado que la glutamina, la asparagina, el glutamato y el aspartato estimulan la fijación de nitrógeno (Kouchi et al, 1991). Estos resultados sugieren que en la relación metabólica entre la planta y la bacteria, pueden estar involucrados los aminoácidos, como la glutamina (Durán & Calderón, 1995; Durán et al, 1995, Huerta-Zepeda et al, 1996; Kahn et al, 1985; Rastogi & Watson, 1991).

Se han reportado una gran cantidad de estudios referentes al transporte de aminoácidos en bacteroides aislados, donde se observa la acumulación de ciertos aminoácidos. Se ha descrito para *R. leguminosarum* la existencia de una permeasa general de aminoácidos (Aap) que transporta una amplia variedad de aminoácidos (glutamato, aspartato, prolina, histidina y aminoácidos alifáticos). Dicho transportador parece ser el principal sistema de regulación de entrada y salida de aminoácidos del bacteroide; sin embargo no es el único: mutantes que no expresan Aap incorporan glutamato, alanina y leucina. (Day et al, 2001; Walshaw & Poole, 1996). Para el caso de la glutamina, no se ha descrito la existencia de un transportador específico, sin embargo consideramos que esa posibilidad aún persiste.

La glutamina y su recambio ocurren activamente en bacteroides, como lo demuestra la acumulación de glutamina y un bajo contenido de glutamato en bacteroides de la mutante LM16, en comparación a las pozas de aminoácidos encontradas en bacteroides de la cepa silvestre (Durán et al, 1995). Es posible que la glutamina sea proporcionada por la planta al bacteroide, en base a la alta actividad de glutaminasa A encontrada en bacteroides y a la relativamente baja actividad de GS's encontrada en los mismos durante la simbiosis.

Si durante el proceso de simbiosis entre *R. etli* y *P. vulgaris* la "moneda metabólica" que reconocen ambos organismos son los aminoácidos, la glutaminasa A puede tener un importante papel en la utilización de la glutamina como fuente de carbono para el bacteroide, proporcionada por la planta, o bien, en el recambio de compuestos nitrogenados a partir de la catálisis de la glutamina como un donador primario de nitrógeno.

En este sentido, recientemente se ha retomado la propuesta de Kahn referente al aporte de aminoácidos de la planta al bacteroide (Lodwig et al, 2003). Se propone que existe un complejo intercambio de aminoácidos entre la planta y el bacteroide, el cual es esencial para la fijación de nitrógeno: la planta provee de glutamato (o posiblemente glutamina) al bacteroide, el cual sintetiza aspartato que es exportado a la planta, para la síntesis de asparagina. Este

intercambio permite que la planta controle el aporte de glutamato al bacteroide en función al aspartato que toma de regreso; mientras que el bacteroide mantiene la relación como mutualista debido a que la planta depende del aporte de aspartato que proviene del bacteroide.

Implicaciones biotecnológicas del ciclaje de la glutamina

Los ciclos de sustrato se han estudiado ampliamente a fin de darles significado biológico. La compleja red metabólica en la que operan los ciclajes dificulta la interpretación de su papel, aún más cuando dicho papel se evalúa en condiciones ambientales controladas y con exceso de nutrimentos, como las provistas en laboratorio (Huerta-Saquero et al, 1998). Los ciclos de sustrato han sido seleccionados como posibles puntos regulatorios del flujo metabólico bajo controles muy estrechos de las enzimas participantes (control alostérico reciproco, modificación covalente, diferentes constantes catalíticas, etc.) a fin de reducir al mínimo la pérdida energética, sin alterar la plasticidad del sistema para responder a cambios en las condiciones metabólicas de manera inmediata (Stein & Blum, 1978).

En este contexto, la ocurrencia del ciclaje de la glutamina en *R. etli* puede justificarse como un mecanismo de adaptación a las condiciones cambiantes del medio, que en condiciones de laboratorio parece una simple pérdida de energía inútil, como ya se discutió en el manuscrito anexo.

La mutante LM16, cuyo gen *glsA* fue interrumpido mediante la inserción de un transposón Tn5, presenta un mayor rendimiento en biomasa producida de un 7 a un 10% respecto al rendimiento encontrado para la cepa silvestre. Estos porcentajes –aparentemente bajos- pueden tener implicaciones biotecnológicas nada despreciables: si la ocurrencia de los ciclajes en microorganismos de interés biotecnológico, -como los utilizados para la obtención de biomasa, para la sobreproducción de aminoácidos, antibióticos,

polímeros, etc.- son interrumpidos mediante mutaciones dirigidas a las enzimas catabólicas participantes en el ciclaje, es posible incrementar su rendimiento siempre y cuando se logre direccionar el ahorro energético hacia las vías metabólicas adecuadas o incluso hacia el crecimiento.

El ciclaje de la glutamina en la naturaleza ¿regla o excepción?

Se ha propuesto la ocurrencia del ciclaje de la glutamina en diferentes microorganismos, como se describió anteriormente. Existe la posibilidad que dicho ciclaje se presente como un mecanismo favorecido evolutivamente. En este sentido, la glutamina es un compuesto clave en el metabolismo celular, y sus vías de síntesis y degradación son similares en prácticamente todos los microorganismos estudiados. La secuenciación de genomas que actualmente son liberados en el GenBank nos permiten determinar la existencia de glutaminasas en diferentes organismos y abren la posibilidad de explorar la ocurrencia del ciclaje de la glutamina en los mismos. Como ejemplo de lo anterior, baste decir que existen alrededor de 70 microorganismos diferentes que presentan en su genoma secuencias de probables glutaminasas, muchos de los cuales presentan genes que codifican para dos isoformas. Las glutaminasas se presentan también en eucariontes, desde unicelulares hasta organismos más complejos como el *Homo sapiens*.

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PAGINACIÓN DISCONTINUA

Regulation of *Rhizobium etli* glutaminase A.

Implications to glutamine-cycling regulation

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[1] This work is dedicated to the loving memory of Dr. Jorge Calderón.

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Abstract

The present study determines the regulatory mechanisms that operate on *Rhizobium etli* glutaminase A. Expression levels of the *glsA* gene under several metabolic conditions were evaluated by fusions of the *glsA* gene promoter and the transcriptional reporter cassette *uidA2-aad*. *glsA* expression was directly correlated to the glutaminase A activity found in the tested growth conditions reaching its maximum level in the presence of glutamine and during the exponential growth phase. Glutamine induces *glsA* expression.

The influence of allosteric metabolites on glutaminase A activity was also determined. The purified enzyme was inhibited by 2-oxoglutarate and pyruvate, while oxaloacetate and glyoxylate modulate it positively. Glutaminase A is not inhibited by glutamate and is activated by ammonium.

Glutaminase A participates in an ATP-consuming cycle where glutamine is continually degraded and resynthesized by glutamine synthetase (GS). GS and glutaminase A activities appear simultaneously during bacterial growth under different metabolic conditions and their control mechanisms are not reciprocal. Slight overproduction in glutaminase A expression causes a reduction in growth yield and a dramatic decrease in bacterial growth.

The determination of the glutaminase A regulatory mechanisms allows to propose that the energetic cost represented by glutamine cycling is justified, since it could be a mechanism that allows bacteria to direct the metabolic flow towards the use of alternative carbon sources (amino acids, particularly glutamine) when other carbon sources are not available, and redirect the metabolic flow towards the biosynthesis of amino acids or other nitrogenous compounds from glutamine, with unlimited carbon source.

1. Introduction

Glutamine plays a central role in the nitrogen metabolism of micro-organisms and is the final product of ammonium assimilation. It is synthesized by glutamine synthetase (GS) from ammonium and glutamate. *Rhizobium etli* assimilates ammonium mainly through the GS-glutamate synthase (GOGAT) pathway [1,2]. In these symbiotic nitrogen-fixing bacteria, three different GS isozymes have been described that differ in their regulation and their role in free-living and symbiotic states: GSI activity is induced when the organism is grown on a rich medium, whereas GSII activity is induced and regulated by nitrogen in minimal medium [1,3]. A third GS has been reported but its physiological role remains unclear [3,4].

In *R. etli*, glutamine is assimilated by transamidation reactions. It can also be converted to 2-oxoglutarate and ammonium by the enzymes of the glutamine transaminase-ω-amidase pathway. *R. etli* glutamine transaminase may play a role in the irreversible synthesis of glycine, alanine and other amino acids [5]. Additionally, in *R. etli*, glutamine is degraded by two glutaminases (EC 3.5.1.2) that catalyse the hydrolytic deamidation of glutamine resulting in the production of glutamate and ammonium. Glutaminase isozymes differ in regulation, electrophoretic mobility and thermostability [6]: a thermolabile glutaminase A, which is positively regulated by glutamine and negatively regulated by the carbon source; and a constitutive and thermostable glutaminase B. Glutaminase A plays a catabolic role in the degradation of glutamine to carbon skeletons and to maintain the optimal balance between glutamine and glutamate, the universal nitrogen donors of the cell. Also, in *R. etli* bacteroids, glutamine is degraded by glutaminase during symbiosis with *Phaseolus vulgaris* [6,7].

The ammonium released from glutamine by glutaminase and transaminase-ω-amidase activities is assimilated by GS, thus leading to the operation of a glutamine cycle where glutamine is continually synthesized and degraded at the expense of ATP [5,6,7,8]. Several functions have been proposed for the glutamine cycle [2,9,10,11,12,13]: a way to rapidly regulate the intracellular glutamine pool and thus control the rate of synthesis and degradation of cellular nitrogen compounds; a general way to take up or release the carbon skeletons of organic nitrogen metabolites; a way of maintaining the optimal balance between glutamine and glutamate; for energy dissipation; to expend energy to induce carbon flux and generate more energy; and to allow continuous ATP usage and drive an effective carbon flow that supports growth.

Although glutamine synthesis regulation has been extensively studied in *R. etli*, less is known about glutamine degradation regulation, despite the fact that glutamine concentration is the result of its synthesis and degradation. Since glutaminase A has the highest glutamine-degrading activity in a variety of growth conditions during exponential phase and in bacteroids -which suggest its activity could play an important role in glutamine cycling and during symbiosis-, it was of interest to determine whether glutaminase A activity is regulated at molecular level, to gain insight into glutamine substrate cycle regulation. The aim of the present work was to determine how glutaminase A is regulated at transcriptional and posttranslational levels.

We presently propose a regulation model of glutaminase A, and its contribution to glutamine cycle regulation is discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium* strains were grown aerobically at 30°C in rich medium (PY) or in minimal medium (MM). The nitrogen and carbon sources in MM were used at a concentration of 10 mM. *Escherichia coli* strains were grown aerobically at 37°C in LB broth. When needed, antibiotics were added at the following concentrations (μg/ml): Nalidixic acid (Nal, 20), Streptomycin (Sm, 200), Kanamycin (Km, 30), Spectinomycin (Sp, 200), Carbenicillin (Cb, 150), Tetracycline (Tc, 10), Cloramphenicol (Cm, 20). Antibiotics, nitrogen and carbon sources and IPTG were purchased from Sigma Chemical Co.

2.2. DNA Manipulations

Recombinant DNA standard techniques were used [14]. Restriction enzymes, alkaline phosphatase and T4 ligase were purchased from GIBCO-BRL. Plasmid DNA was isolated from *E. coli* by standard procedures and mobilized into *R. etli* cells by conjugal matings.

2.3. Transcriptional fusions glsA-GUS

Transcriptional fusions with the *uidA2-aad* cassette (from plasmid pWM5) and *glsA* promoter were constructed. *uidA2* encodes for GUS, which catalyzes the hydrolysis of β-glucuronides. *uidA2-aad* cassette [15] was obtained by *Sac*I digestion of pWM5 and cloned into unique *Sac*I site in the *glsA* structural gene contained in pLAFR1 cosmid (pCD2.1) [16]. The *Sac*I site is 90 bp downstream from the *glsA* ATG start codon. The new constructions have 555 bp preceding the *glsA* structural gene which presumably contains the *glsA* promoter region as indicated by the glutaminase activity regulation found in CE3/pCD2.1 and LM16/pCD2.1 strains similar to that found in the CFN42 wild-type strain previously reported by Calderón et al [16]. We selected two constructions that differ in their orientation from the *glsA* promoter, one in the same orientation (p*glsA*/GUS) and the second anti-sense (p*glsA*/SUG), used as a control. The new constructions were mobilized into *R. etli* CE3 and LM16 cells by conjugal matings.

2.4. β-Glucuronidase activity assay

β-Glucuronidase assays employed 4-methyl-umbelliferyl-β-D-glucuronide (Sigma) as a substrate along with sonicated cell extracts as described elsewhere [17]. Samples of 100 μl were taken at three time points between 5 and 20 min and then mixed with 900 μl of stop buffer (0.2 M Na_2CO_3). Fluorimetric determinations were made with a Perkin- Elmer LS-5 (excitation

wavelength 360 nm; emission wavelength 446 nm). The fluorimeter was calibrated with 4-methylumbelliferone standards. Specific enzyme activity in cell extracts was expressed as nanomoles of 4-methylumbelliferone min^{-1} mgP^{-1}. Protein content was determined by Bradford assays [9].

2.5. Purification of R. etli glutaminase A

Recombinant (His_6)-glutaminase A produced in *E. coli JM109/pTAHS* was purified to homogeneity by metal-affinity chromatography as previously described [18].

2.6. Determination of glutaminase and GS activities

Glutaminase activity was assayed as described elsewhere [18]. Synthethase activity of GS was assayed at pH 7.6 as described by Bender et al [19].

2.7. Posttranslational regulation of glutaminase A

We explored the possible influence of allosteric effectors on enzyme activity of purified glutaminase A. Adenine nucleotides ATP, ADP, AMP, NAD^+ and NADH (from 0.1 to 10 mM); glutamate, ammonium, tricarboxylic acid intermediates (2-oxoglutarate, succinate, fumarate, malate, oxaloacetate), pyruvate, glyoxylate, and divalent cations (Ca, Mg, Mn) (from 0.1 to 50 mM), were evaluated as allosteric effectors.

2.8. Growth yield and succinate consumption

Succinate consumption was measured employing cell-free MM from 20 h growth bacterial strains as a substrate for succinate dehydrogenase activity. Oxygen-consumption by succinate dehydrogenase activity was proportional to non-degraded succinate of growth media and was measured by an oxymeter. Reaction buffer was Sucrose-Hepes-EGTA (SHE, pH 7.6) and sodium dithionite was employed as a standard. Succinate dehydrogenase enzyme was obtained from partially-purified heart-tissue sub mitochondrion particles. Succinate consumption was calculated by subtracting non-degraded succinate to initial succinate supplemented to growth media.

Bacterial growth was monitored spectrophotometrically at 540 nm and measured by total-protein determination according to the method by Lowry et al [20].

2.9. Overproduction of R. etli glutaminase A and E. coli glutaminase A

We cloned the *glsA* gene from *R. etli* (that encodes for glutaminase A, 18) and the *glsA* gene from *E. coli* (that encodes for glutaminase A) in the wide-host-range pMMB206 cloning vector (Table 1). pMMB206 has an hybrid promoter Tac-lacUV5 that allows controlled expression of glutaminases *in vivo*. The *R. etli glsA* gene was amplified by PCR with oligonucleotides that

introduce new restriction enzyme sites at both sides of the structural gene (*Bam*H1 and *Hind*III sites, respectively; 18). The *E. coli* glutaminase A gene amplified by PCR was cloned into pUC18 *Bam*H1-*Eco*R1 sites. (Oligonucletides 11 and 12). The cloned gene was transferred to pMMB206 into *Bam*H1-*Hind*III sites. The designed oligonucleotides were named as follows: (5' to 3'): 11.-CGCGGA TCCATG TTAGAT GCAAAC; 12.-CGGAAT TCAGCC CTTAAA CACG.

Growth determinations of CE3 (WT) and LM16 mutant and of both strains carrying p6*glsA* or p6*glsA*coli were done in PY and MM. For MM we tested glutamine-succinate, ammonium-succinate and glutamine as nitrogen and carbon source, with and without IPTG as inducer. GS and glutaminase activities were determined for each strain.

3. Results

3.1. Transcriptional regulation of glutaminase A

To evaluate the trascriptional regulation of *glsA*, we constructed transcriptional fusions with the *uidA2-aad* cassette and *glsA* promoter. GUS and glutaminase enzymatic activity determinations were performed for CE3, LM16, CE3p*glsA*/GUS, LM16p*glsA*/GUS, and the antisense CE3p*glsA*/SUG and LM16p*glsA*/SUG *R. etli* strains. All strains were grown for 16 or 24 h in MM with ammonium-succinate, glutamine-succinate or glutamine as nitrogen and carbon sources. GUS activity of CE3p*glsA*/GUS for the different growth conditions was directly correlated with glutaminase activity of the same strain (Fig. 1). The expression of *glsA* reached maximal values in the presence of glutamine and during the exponential growth phase, while its expression decreased in the stationary phase. LM16p*glsA*/GUS showed similar activity values as CE3p*glsA*/GUS, except in glutamine as sole carbon and nitrogen source, where it did not grow (data not shown). Strains with the anti-sense constructions (CE3p*glsA*/SUG and LM16p*glsA*/SUG) exhibited activity below 20 nmoles $min^{-1}mgP^{-1}$, for all conditions (not shown).

The expression of *glsA* (GUS) under different growth conditions correlated with glutaminase A activity as shown in Table 2. Maximum glutaminase A activity and *glsA* expression, taken as 100%, were shown in media with glutamine as sole carbon and nitrogen source. With glutamine as nitrogen source, *glsA* expression was high independently of the carbon source. With glutamate as sole carbon and nitrogen source, glutaminase A activity is similar to that observed in glutamine, while GUS expression is 30% lower, which could be due to glutamate confers stability to the enzyme as we showed previously [18]. Glucose exerted a negative influence in

both activities in comparison to succinate as carbon source. Glutaminase A activity and GUS expression under the tested growth conditions were considerable suggesting that glutaminase A activity is important for the metabolic turnover of nitrogenous compounds (for instance, amino acids) during the exponential growth phase, and to mantain the optimal balance between glutamine and glutamate as we reported previously [5]

3.2. Allosteric control of R. etli glutaminase A

Glutaminase A was purified to homogeneity by recombinant methods [18]. The kinetic parameters were determined (Km and Vm) and the influence of probable allosteric modulators, tested. The products of glutaminase A activity -glutamate and ammonium– did not inhibit enzymatic activity; moreover, ammonium activates glutaminase A (Ka=1 mM). Glutaminase A was not modulated by ATP, ADP, AMP, NAD^+, NADH, or divalent cations, as opposed to *E. coli* glutaminase B activity, which is altered by the presence of adenine nucleotides and divalent cations [25]. Metabolites that influence purified glutaminase A activity are shown in Table 3. Important inhibition is exerted by 2-oxoglutarate and pyruvate (60 and 50% respectively) , while oxaloacetate and glyoxylate behave as positive modulators of the enzyme (increasing its activity by 30%). The sigmoidal kinetic behavior of glutaminase A suggests cooperativity between subunits, and its analysis resulted in a Hill coefficient of 2 (not shown).

3.3 In vivo controlled expression of R. etli glutaminase A and E. coli glutaminase A in CE3 and LM16 strains.

Glutamine cycling suggests an important loss of energy due to glutamine degradation by glutaminase A and glutamine re-synthesis via GS. This energetic cost could affect growth yield or bacterial fitness unless the participating enzymes were subject to transcriptional and/or posttranslational antagonistic control. In this sense, the comparative growth yield of CE3, LM16 and overexpressed glutaminase A strains provided information about control mechanisms and the energetic cost due to the overexpression of glutaminase A activity. Considering that the effect in bacterial yield and fitness in enzyme-overexpressed strains (i.e. glutaminase A) could be due to overexpression *per se* and not only to enzymatic activity, we constructed the plasmid p6*glsA*coli (pMMB206 harbouring the *E. coli* glutaminase A structural gene) as overexpression control (Table 1). *E. coli* glutaminase A has similar size to *R. etli* glutaminase A (310 and 309 amino acids, respectively). *E. coli* glutaminase A is active at pH 5, while at physiological pH (6.8-7.2) it is inactive.

Growth kinetics were determined of CE3, LM16, CE3p6*glsA*, LM16p6*glsA*, CE3p6*glsA*coli and LM16p6*glsA*coli strains in PY and MM with ammonium-succinate, glutamine-succinate and glutamine as carbon and nitrogen source. In PY and glutamine-succinate, strain growth did not show significant differences. In MM with glutamine as sole carbon and nitrogen source, CE3, CE3p6*glsA* and CE3p6*glsA*coli strains showed no significant differences in growth. LM16 does not grow on glutamine as sole carbon and nitrogen source because it has no glutaminase A activity [5,6]. LM16p6*glsA*coli, despite containing the *E. coli* glutaminase A plasmid, did not grow on that medium: glutaminase activity was determined, and LM16p6*glsA*coli showed high glutaminase activity at pH 5, which was undetectable at physiological pH (it can therefore not complement the lack of *R. etli* glutaminase A) (data not shown). In MM with ammonium-succinate as nitrogen and carbon source, growth of CE3, LM16, CE3p6*glsA* and CE3p6*glsA*coli strains did not reveal significant differences (Fig. 2, panel A); on addition of 0.05 mM IPTG as inducer of the expression of the genes *glsA* (*R. etli* glutaminase A) and *glsA*coli (*E. coli* glutaminase A) an important reduction is observed in the growth of CE3p6*glsA* strain, but not of CE3p6*glsA*coli (Fig. 2, panel B). The energy expenditure of strains with plasmid pMMB206 do not represent observable influence on growth. Also, the induction of *E. coli* glutaminase A in CE3p6*glsA*coli, as well as in CE3pMMB206 and LM16pMMB206 did not show significant differences in growth with respect to CE3 and LM16, while the decrease of nearly 50% in growth of CE3p6*glsA* strain may be ascribed to overexpression of glutaminase A activity, which causes glutamine degradation to be very efficient with the concomitant rise in re-synthesis via GS, with ATP and reducing power consumption.

The differences found in the growth kinetics of these strains lead us to determine their growth yield (Table 4). CE3, LM16, CE3p6*glsA* and CE3p6*glsA*coli strains were grown in MM with ammonium-succinate as carbon and nitrogen source for 20 h, with and without IPTG as overexpression inducer. Total protein was quantified as growth parameter and succinate consumption as indicated in Materials and Methods. The ratio between succinate consumed and micrograms of total protein produced was taken as the yield, considering CE3 as 100%. When strains were grown on ammonium-succinate no significant differences were observed in the yield of CE3 and CE3p6*glsA*coli. LM16 strain showed a slight but constant increment of 7%, while CE3p6*glsA* showed a decrease of 14% with respect to CE3 even in absence of IPTG (Table 4). Plasmid pMMB206 has a low copy number and its basal expression is low because it contains the

gene that codes for the repressor *lacI*^{q} (26). When grown on ammonium-succinate + 0.05 mM IPTG, LM16 showed a 12% higher yield than CE3, while a much larger decrement in yield was observed in CE3p6*glsA* of up to 42% with respect to CE3. Succinate consumption of CE3p6*glsA* and CE3p6*glsA*coli strains was equivalent and slightly lower to that of CE3 (178, 172 and 200 µmoles succinate ml^{-1} respectively) while total protein production of CE3p6*glsA* was clearly lower (40 vs 78 µgP ml^{-1} of CE3).

3.4. GS and glutaminase activity of CE3, LM16, CE3p6glsA and CE3p6glsAcoli strains

Differences between bacterial growth of CE3 and CE3p6*glsA* in the induction of glutaminase A overproduction (Fig. 2 panel B), as well as differences in their growth yield (Table 4) suggests an increase in the speed of break down and re-synthesis of glutamine, with the consequent energetic cost which translates as low yield in CE3p6*glsA* strain. Therefore, glutaminase and GS enzymatic activities were determined of CE3, LM16, CE3p6*glsA* and CE3p6*glsA*coli grown for 20 h in ammonium-succinate, with and without IPTG.

Glutaminase and GS enzymatic activities of the different strains are shown in Table 5. The lack of glutaminase A in LM16 is reflected by its low glutaminase activity, which does not alter the GS activity when compared with activity found in CE3. CE3p6*glsA* without inducer shows three times more glutaminase activity than the wild type, which does not have an effect on GS activity either. However, overexpression of glutaminase results in 15 times higher activity, which causes an important increase in GS activity (from 3 to 15 nmoles min^{-1} mgP^{-1}). This shows that when glutamine catalysis is increased, its synthesis is increased with the consequent energy cost which translates as a dramatic decrease in growth (Fig. 2). Overexpression of *E. coli* glutaminase A did not produce significant changes in enzymatic activity of glutaminase or GS (Table 5)

4. Discussion

In *R. etli*, ammonium assimilation and glutamine synthesis is carried out by the GS-GOGAT pathway and glutamine is degraded by various enzymes, among them glutaminase A, which gives origin to a futile cycle of simultaneous synthesis and break down of glutamine with ATP consumption [8]. It has been suggested that glutamine cycling is necessary to maintain the continuous use of ATP to generate an effective flow of carbon for growth [27,28]. The interaction between carbon metabolism and glutamine synthesis has been shown: in glutamine-succinate, glutamine synthesis favours the use of succinate, while in glutamine –where it is present in excess– its synthesis is restricted and it is used as source of energy [5,8]. However, in

ammonium-succinate, glutamine cycling has no apparent function. Glutamine cycling is a very attractive model of metabolic regulation, in which glutamine biosynthesis and the regulation of GS's has been extensively characterized, however, the catabolic pathway has not been studied, and this is why we investigated the regulatory mechanisms that operate on glutaminase A. Glutaminase A is the enzyme with the greatest glutamine catalysis capacity; it is indispensable for use of glutamine as carbon source and to maintain the intracellular balance of the universal donors of nitrogen, glutamine and glutamate [5].

Enzymatic activity of glutaminase A varies under different culture conditions, the highest being in presence of glutamine and during the exponential growth phase (Fig. 1). Expression of *glsA* is induced by the presence of glutamine in the culture medium and varies in the different growth phases in direct correlation with glutaminase A activity (Fig. 1). It is important to mention that *glsA* expression is largely present under all tested growth conditions, which suggests that glutaminase A is a key enzyme in the nitrogen metabolism of the bacteria (Table 2). Maximal induction of *glsA* was observed in glutamine as sole carbon and nitrogen source, a condition in which glutamine synthesis is inhibited and, thus, cycling is reduced allowing the use of the glutamine carbon skeletons while ammonium excess is secreted to the culture medium (5,8). In glutamine-succinate, *glsA* expression as well as enzymatic activity of glutaminase A is also high, which allows the use of glutamine as carbon and nitrogen source, although it has been demonstrated that *R. etli* oxidizes succinate in preference to glutamine [8]. In this growth condition, GSII is induced, thus, glutamine cycling is proposed as a reaction to dissipate energy, that helps drive the utilization of succinate [8,12,29,30]. In ammonium-succinate, *glsA* expression and glutaminase A enzymatic activity are lower compared to the previous conditions; in spite of this, glutamine synthesis and break down occur simultaneously as shown by Encarnación et al [8]. The presence of glutaminase A in these conditions has no apparent significance but the useless expenditure of energy, unless regulatory mechanisms exist that maintain the enzymatic activity of glutaminase A at basal levels or there is antagonistic control between GS and glutaminase A activities. Considering the need to define the mechanisms that regulate the glutaminase A enzymatic activity, we determined the influence of possible allosteric modulators.

Inhibition by 2-oxoglutarate and pyruvate (Table 3) suggests a strong relationship between carbon metabolism and glutamine utilization. When the amount of available carbon increases, the concentration of pyruvate and 2-oxoglutarate does so as well and this inhibits glutaminase A

activity allowing glutamine to be used by an alternative pathway (for example, the glutamine-ω-amidase transaminase pathway) as nitrogen donor, using 2-oxoacids for the synthesis of amino acids, as in *S. cerevisiae* [31]. On the other hand, when the amount of available carbon decreases, GS activity falls and the withdrawal of TCA cycle intermediates for anabolism should be replaced. Rhizobia contain a variety of anaplerotic enzymes which function in this capacity [28]. In this sense, oxaloacetate and glyoxylate could be incorporated to TCA cycle by aspartate catabolism and the glyoxylate bypass, respectively [32]. Concomitantly, glutaminase activity increases to allow glutamine can be used as carbon source. Activation of glutaminase A by ammonium allows bacterial growth on glutamine, a condition in which large amounts of ammonium are secreted [5]. Evaluation of the growth yield of CE3 and of LM16 in ammonium-succinate revealed a yield for LM16 greater in 7% with respect to the WT, which suggests that glutamine cycling implies an important energy cost for bacteria. If we manipulate glutamine cycling by overexpression of the glutaminase A activity under the control of an inducible promoter, we can overcome the regulatory mechanisms of the enzyme at transcriptional and allosteric level, which allows us to amplify the energetic cost of cycling. An increase in the expression of glutaminase A causes a rise in GS activity in the strain CE3p6*glsA* (Table 5) and a dramatic decrease in growth (Fig. 2). The fall in growth of CE3p6*glsA* is specifically attributed to the elevated glutaminase A activity, and not to overexpression *per se*, since it was used as control *E. coli* glutaminase A overexpression, which was cloned and overexpressed in the same vector. *E. coli* glutaminase A is not active at physiological pH, therefore, cycling did not increase nor present significant influence on the yield of CE3p6*glsA*coli with respect to the WT (Table 5, Fig. 2).

The habitat of *R. etli* is soil and it can establish a symbiotic relationship with *P. vulgaris*. In free life, nutrient conditions may vary importantly and bacteria must therefore maintain a metabolic plasticity that allows them to confront these changing conditions, adapting as quickly as possible to the new metabolic conditions. In this context, we propose glutamine cycling could be a mechanism that allows bacteria to direct their metabolic flow towards the use of alternative carbon sources (amino acids, specifically glutamine) when other sources are not available or are limiting; or else, when the carbon source is not a limiting factor, to redirect the metabolic flow towards the biosynthesis of amino acids or other nitrogen metabolites from glutamate or glutamine (Fig. 3). If carbon sources (as succinate or glucose) are available there is a rise in

pyruvate and 2-oxoglutarate, which inhibits the break down of glutamine by glutaminase A and allows it to be used in the biosynthesis of amino acids and other nitrogen compounds. If the carbon source becomes the limiting factor, glutamine can be used as carbon source and glutaminase A expression is induced, enzymatic activity increases and this leads to bacterial growth.

Glutamine cycling caused an energy loss that, under optimal nutritional conditions, such as those provided in the laboratory, does not imply important physiological significance; however, it could be selected to allow bacteria to adapt to the changes in nutritional conditions, in spite of the energetic cost it represents.

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TABLE 1. Bacterial strains and plasmids used in this study

Strains	Relevant phenotype	References
E. coli		
S17-1	*recA, endA, thi, hsdR,* RP-2-Tc::Mu::Tn7, Tp^r, Sm^r	21
JM109	*recA1, relA1, supE44,* Δ(*lac-proAB*), [F', *traD*36, *proAB, lacI*^{q}ZΔ*M15*]	22
R. etli		
CFN42	Wild type. Nal^r	23
CE3	Derivative of CFN42 wild type. Nal^r, Sm^r	23
LM16	Derivative of CE3. *glsA*::Tn5. Nal^r, Sm^r, Km^r	7
Plasmids		
pTrcHis	His-tag expression vector. Amp^r, Cb^r	Invitrogen
pWM5	Derivative of pUC1318. Ω *nidA2-aadA*. Sp^r, Sm^r	15
pLAFR1	Wide-host-range cloning cosmid. *mob*$^+$, *tra*$^-$, *Inc*P^+, Tc^r	24
pMMB206	Wide-host-range cloning vector. Cm^r	22
pCD2.1	2.1 Kb *Hind*III fragment containing *R. etli glsA* gene in pLAFR1	16
p*glsA*/GUS	Derivative of pCD2.1. *glsA :: nidA2-aadA*	This work
p*glsA*/SUG	The same as p*glsA*/GUS anti-sense *nidA2-aadA* cassette	This work
p6*glsA*	Derivative of pMMB206. Ω *R. etli glsA* gene.	This work
p6*glsA*coli	Derivative of pMMB206. Ω *E. coli glsA* gene.	This work

TABLE 2. Glutaminase A and β-glucuronidase activities of CE3p*glnA*/GUS strain in different growth conditions

Nitrogen and carbon source*	Glutaminase A activity (%)[c]	β-glucuronidase activity (%)[c]
Glutamine	100	100
Glutamine-Succinate	80	88
Glutamine-Glucose	60	72
Ammonium-Succinate	55	70
Glutamate	100	70
Nitrate-Succinate	75	67
Glutamate-Succinate	80	60
Aspartate-Succinate	80	60

*10 mM each on MM.

Activity from protein extracts of strains after 16 h of growth.

[c] Maximum activity for both enzymes was found in glutamine as a sole carbon and nitrogen source and was considered as 100%.

TABLE 3. Allosteric effectors of *R. etli* glutaminase A

Effector	Conc[c]	Modulation	% of modulation*
Oxaloacetate	10	Positive	30 (5)
Glyoxylate	10	Positive	30 (8)
2-oxoglutarate	10	Negative	60 (13)
	1		30 (6)
	0.1		20 (7)
Pyruvate	10	Negative	50 (10)
	1		20 (4)
Fumarate		Negative	30 (9)

[c] Effector concentration in mM.

* Percentage of specific activity increment/decrement in reference of CE3 dialyzed extract without effectors. SD is shown in parenthesis.

TABLE 4. Growth yield and succinate consumption of *R. etli* strains

Strain	Growth condition	µgP ml^{-1}	Succinate consumption*	µmoles succ µgP^{-1}	Yield (%)
CE3	Ammonium-Succinate	84 (7)	182 (8)	2.17	100
LM16		82 (4)	166 (5)	2.02	107
CE3p6*glsA*		67 (10)	169 (4)	2.52	86
CE3p6*glsA*coli		81 (6)	176 (11)	2.17	100
CE3	Ammonium-succinate + 0.05 mM IPTG	78 (5)	200 (8)	2.56	100
LM16		71 (6)	162 (18)	2.28	112
CE3p6*glsA*		40 (9)	178 (11)	4.45	58
CE3p6*glsA*coli		67 (6)	172 (17)	2.57	99

*Expressed as µmoles succinate ml^{-1}. All determinations were done from protein extracts of strains after 20 hours of growth in MM. Standard errors are shown in parenthesis.

TABLE 5. Glutaminase and GS activities of *R. etli* strains

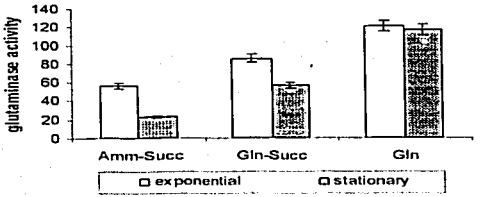
	Glutaminase activity[a]		GS activity[b]	
Strain		+ IPTG		+ IPTG
CE3	58	56	2	2
LM16	10	8	2	3
CE3p6glsA	146	827	3	15
CE3p6glsAcoli	57	59	3	3

[a] Expressed as nmol ammonium min^{-1} mgP^{-1}.

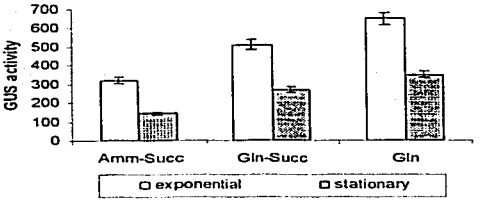
[b] Expressed as nmol γ-glutamyl hydroxamate min^{-1} mgP^{-1}.

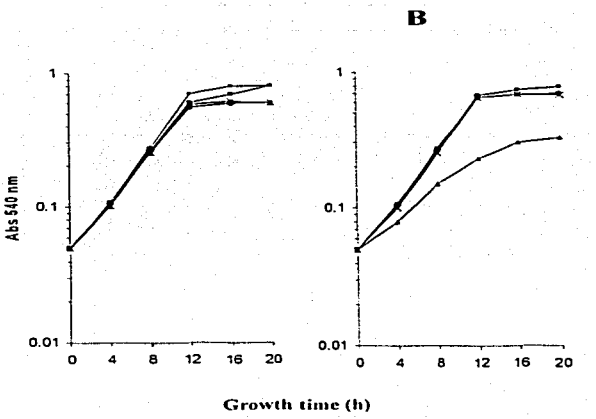
All determinations were done from protein extracts of strains after 20 hours of growth in ammonium-succinate. The data represent the means of at least three different determinations, with a standard deviation of < 5 %.

A

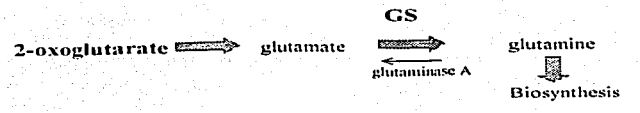


B





a) Available carbon:



b) Limited carbon:

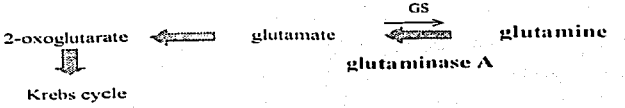


FIGURE LEGENDS

FIG. 1. (A) Glutaminase and (B) β-glucuronidase activities of *R etli* CE3p*glsA*/GUS. Enzymatic activities were determined from bacterial extracts grown at 16 h (exponential phase) and 24 h (stationary phase) in three different MM. Both activities are expressed as nmoles min^{-1} mgP^{-1}

FIG. 2. Growth kinetics of ● CE3, ■ LM16, ▲ CE3p6*glsA* (carrying *R. etli* glutaminase A) and × CE3p6*glsA*coli (carrying *E. coli* glutaminase A) strains. Each strain was grown aerobically in A) ammonium-succinate or B) ammonium-succinate + 0.05 mM IPTG. Growth kinetics were done at least three times. Representative results are shown.

FIG. 3. Metabolic flow of glutamine utilization

a) With carbon availability, the flow is directed to the biosynthesis of nitrogen compounds.

b) In case of limited carbon, the flow is directed towards the Krebs cycle.