UNIVERSIDAD MICHOACANA DE SAN NICOLAS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUIMICO BIOLOGICAS

CARACTERIZACION DE GERANIL-CoA Y 3-METILCROTONIL-CoA CARBOXILASAS DE *Pseudomonas aeruginosa* INVOLUCRADAS EN LA DEGRADACION DE LEUCINA E ISOPRENOIDES ACICLICOS

TESIS QUE PRESENTA:
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I. RESUMEN

La degradación de hidrocarburos (*n*-alcanos) mediada por microorganismos se efectúa mediante las enzimas de la oxidación de ácidos grasos, sin embargo, se requiere de rutas catabólicas alternas cuando estos hidrocarburos presentan ramificaciones en su estructura. El isoprenoide citronelol y el aminoácido leucina son compuestos ramificados que requieren estrategias similares para su catabolismo. Se ha observado que la ruta catabólica de isovalerato y leucina utilizada por *Pseudomonas aeruginosa*, que degrada a estos compuestos hasta acetoacetato y acetil-CoA, presenta similitud con la ruta usada para degradar citronelol, ya que algunas de las actividades enzimáticas presentes en ambas rutas son análogas. Además, se ha encontrado que el metabolito final de la ruta catabólica de citronelol, el 3-metilcrotonil-CoA (MC-CoA), es canalizado por las enzimas del catabolismo de leucina para convertirlo a acetoacetato y acetil-CoA como productos finales. Por lo que se establece que la ruta catabólica de leucina complementa el catabolismo de isoprenoides acíclicos, degradando ambos compuestos hasta acetoacetato y acetil-CoA. En el catabolismo de isoprenoides acíclicos en *Pseudomonas citronellolis*, las enzimas geranil-CoA carboxilasa (GCCasa) y 3-metilcrotonil-CoA carboxilasa (MCCasa), del catabolismo de leucina, han sido consideradas como las enzimas claves de las rutas catabólicas. En este trabajo se determinó que las enzimas GCCasa y MCCasa de *P. aeruginosa* PAO1, se encuentran codificadas por los genes *atuCF* y *liuDB*, respectivamente. Además, se encontró evidencia de que ambas carboxilasas pueden intervenir en las dos rutas metabólicas. Estas dos enzimas fueron expresadas en *Escherichia coli* como proteínas recombinantes, purificadas por cromatografía de afinidad, y posterior a un proceso de desnaturalización y renaturalización, se determinaron los parámetros cinéticos de las enzimas MCCasa y GCCasa recombinantes. Ambas enzimas presentaron valores de pH y temperatura óptimos de 8.5 y 37ºC, respectivamente, la MCCasa mostró una cinética sigmoidal con un $K_{0.5}$ de 9.8 µM, 13 µM y 0.8 µM para 3-metilcrotonil-CoA (MC-CoA), ATP y bicarbonato, respectivamente, no exhibió carboxilación de geranil-CoA (G-CoA). Por otro lado, la GCCasa presentó actividad sobre el G-CoA y el MC-CoA, con una cinética sigmoidal sobre el G-CoA y el bicarbonato con $K_{0.5}$ de 8.8 µM y 1.2 µM, respectivamente, y cinética de Michaelis-Menten con una K_m de 14 µM para MC-CoA y 10 µM para ATP. Las eficiencias catalíticas de la enzima GCCasa sobre G-CoA es de 56 y de 22 para MC-CoA, lo que nos

indica que G-CoA es preferido como sustrato. Se purificó la MCCasa nativa de *P. aeruginosa* PAO1 y se determinaron algunos de sus parámetros cinéticos, en todos los casos los valores fueron similares. La bifuncionalidad de la GCCasa sugiere la probable sustitución de la MCCasa en la ruta catabólica de leucina.

II. ABSTRACT

Hydrocarbon (*n*-alkanes) degradation by microorganisms occurs via the fatty acids oxidation enzymes; however, if the hydrocarbon has a branched-chain structure different catabolic pathways are necessary. The isoprenoid citronellol and the amino acid leucine are branched-chain compounds. Leucine and isovalerate catabolic pathway used by *Pseudomonas aeruginosa* to produce acetoacetate and acetyl-CoA as final products is similar to the citronellol catabolic pathway in the same bacteria, and share analogous enzymes in both pathways. Moreover, 3-methylcrotonil-CoA (MC-CoA), the end product of the citronellol degradation, is taken by the leucine catabolic enzymes to produce acetoacetate and acetyl-CoA. The leucine degradation pathway complements the acyclic isoprenoids catabolism and drives both of them to acetoacetate and acetyl-CoA. The geranyl-CoA carboxylase (GCCase) enzyme from *P. citronellolis*, in the acyclic isoprenoid catabolism, and the 3-methylcrotonil-CoA carboxylase (MCCase) in the leucine and isovalerate catabolism, both have been considered as key enzymes in their respective pathways. These enzymes are encoded by the *atuCF* and *liuDB* genes, respectively, in the *P. aeruginosa* PAO1 chromosome. Furthermore, there is evidence indicating that both enzymes probably have activity in the two pathways. The proteins were expressed in *Escherichia coli* as recombinant proteins, before affinity-chromatography purification, a denaturalization and renaturalization procedures were undertaken to obtain functional enzymes. The two carboxylases showed pH and temperature optima of 8.5 and 37ºC, respectively. MCCase showed a sigmoidal kinetics with a $K_{0.5}$ values of 9.8 μM, 13 μM and, 0.8 μM for 3-methylcrotonil-CoA (MC-CoA), ATP and bicarbonate, respectively. MCCase did not show activity over geranyl-CoA (G-CoA). On the other hand, GCCase had activity with G-CoA and MC-CoA, with a sigmoidal kinetics pattern with G-CoA and bicarbonate showing $K_{0.5}$ values of 8.8 μM and 1.2 μM, respectively, and a Michaelis-Menten kinetics with a K_m of 14 μM for MC-CoA and 10 μM for ATP. The GCCase catalytic efficiency was 56 with G-CoA and 22 with MC-CoA; these values pointed out the G-CoA preference over MC-CoA of this enzyme. The wild type MCCase from *P. aeruginosa* PAO1 was purified and the kinetic parameters were determinated. In all cases they were the same with the recombinant enzyme. The bifunctional activity of the enzyme GCCase suggests a probable substitution of the enzyme MCCase in the leucine catabolic pathway.

III. INTRODUCCION

Los hidrocarburos son compuestos orgánicos ubicuos que están formados de un esqueleto de átomos de carbono a los que se unen átomos de hidrógeno por enlaces covalentes. Algunos hidrocarburos como los alcanos, los alquenos, los terpenos y los compuestos aromáticos son producidos por los procesos biológicos, mientras que otros son producidos en la naturaleza mediante procesos abióticos.

3.1. Degradación microbiana de alcanos

Los hidrocarburos pueden ser utilizados por los organismos como fuente de carbono y energía; sin embargo, la velocidad a la que son consumidos depende de la estructura de la molécula, así como también de la versatilidad metabólica del organismo. Por ejemplo, los alcanos lineales pueden ser catabolizados por los microorganismos mediante las enzimas involucradas en la oxidación de ácidos grasos (degradación β); el único requisito previo consiste en la oxidación de uno de los extremos de la cadena hidrocarbonada para la obtención del ácido carboxílico correspondiente y así poder ser reconocido por las enzimas de esta ruta metabólica. Este tipo de reacciones enzimáticas se ha estudiado extensamente, principalmente en microorganismos. El organismo degradador de alcanos más estudiado es la bacteria *Pseudomonas putida*, que es capaz de efectuar la degradación de *n*-alcanos a través de las actividades enzimáticas codificadas tanto en el plásmido denominado OCT como en el cromosoma, agrupadas en dos regiones dentro del plásmido, la primera forma al operón *alkBFGHJKL* y la segunda constituida por los genes *alkTS* (Witholt *et al.,* 1990; van Beilen *et al.,* 2001). Las enzimas codificadas en este operón intervienen en el reconocimiento, transporte, activación y oxidación del alcano. Las funciones enzimáticas identificadas en este operón son: alcano hidroxilasa (AlkB), dos rubredoxinas (AlkFG), una alcohol deshidrogenasa (AlkJ), una aldehído deshidrogenasa (AlkH), una acil-CoA sintetasa (AlkK), una proteína de función desconocida (AlkL), *alkST* codifica para el tercer componente del sistema alcano hidroxilasa: rubredoxin reductasa (AlkT y AlkS). El modelo del sistema degradativo se presenta en la figura 1.

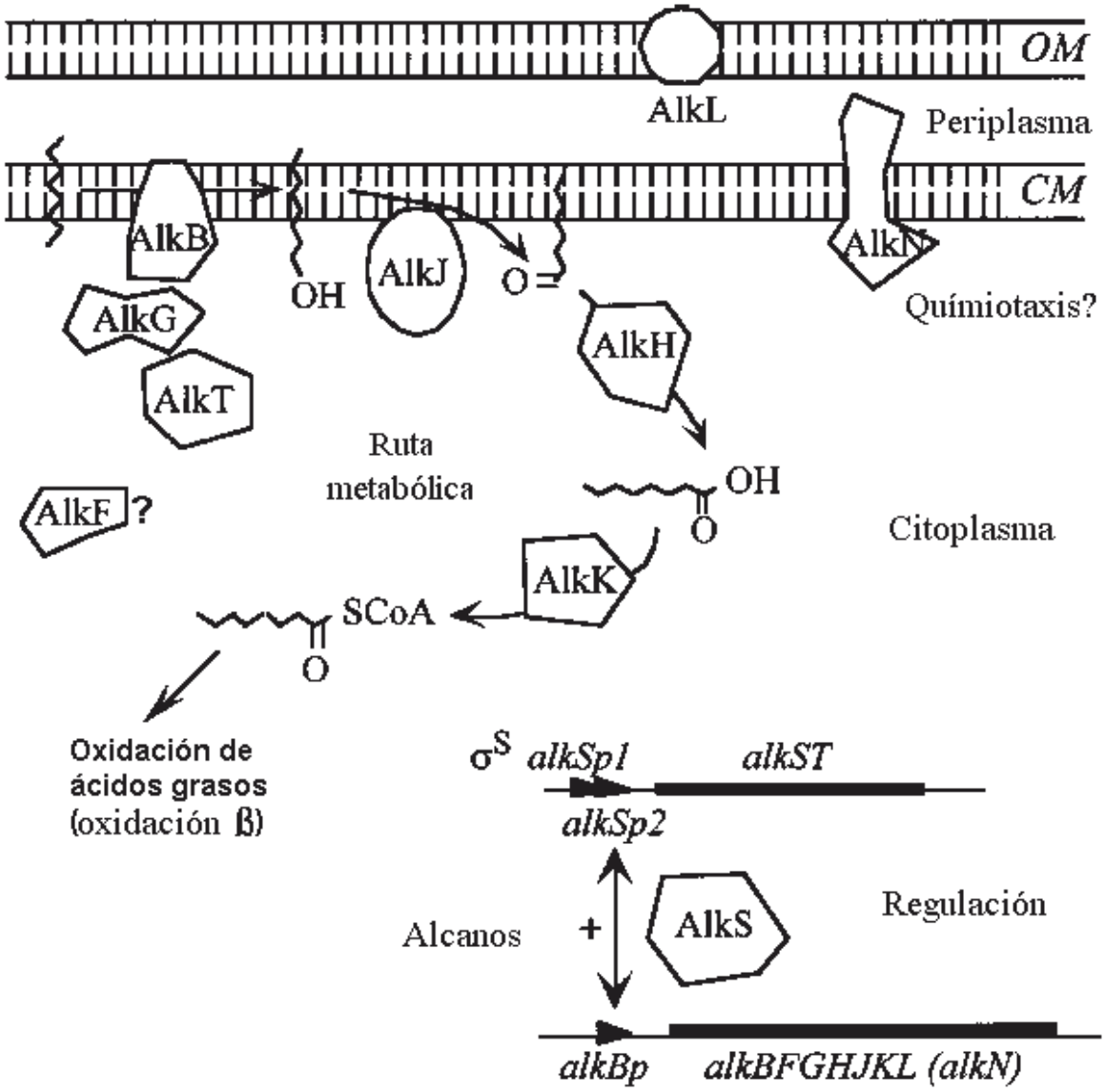


Figura 1: Ruta metabólica de la degradación de alcanos. El operón *alkBFGHJKL* codifica para la enzima alcano hidroxilasa (AlkB), dos rubredoxinas (AlkF y AlkG), una alcohol y una aldehído deshidrogenasa (AlkJ y AlkH, respectivamente), una acil-CoA sintetasa (AlkK), y una proteína de membrana de función desconocida (AlkL). El locus *alkST* codifica para el tercer componente del sistema alcano hidroxilasa, la rubredoxina reductasa (AlkT) y AlkS que regula positivamente la expresión del operón *alkBFGHJKL* y *alkST*. AlkN probablemente es un transductor quimotáctico para alcanos (Tomado de van Beilen *et al*., 2001)

Una vez formado el acil-CoA correspondiente (Figura 1), producto de estas reacciones, este compuesto es direccionado para ser degradado por las enzimas de la oxidación β, usualmente codificadas en el cromosoma bacteriano.

3.2. Degradación de alcanos ramificados

Los hidrocarburos lineales no se consideran como un problema por su persistencia en el ambiente, ya que existe una gran variedad de microorganismos capaces de asimilarlos como fuente de carbono y energía. No ocurre así para los alcanos ramificados, los cuales pueden ser clasificados en dos clases, en base a la resistencia que presentan a la degradación microbiana. Los que cuentan con ramificaciones en el carbono 2 ó carbono α, y aquellos que presentan la ramificación en el carbono 3 ó β. Uno de los alcanos α-ramificados más analizados es el pristano (2, 6, 10, 14 tetrametilpentadecano), que puede ser utilizado por la bacteria *Brevibacterium erythrogenes* como única fuente de carbono y energía, para lo que hace acopio de varias rutas catabólicas que, en conjunto con la oxidación β, permiten la mineralización de dicho compuesto. Al igual que los alcanos lineales, el primer paso para el metabolismo de los alcanos α-ramificados es la oxidación de un extremo de la molécula (oxidación α); el segundo paso es conocido como oxidación ω (se oxida el extremo opuesto de la molécula), produciendo un ácido dicarboxílico (ácido pristandióico) como se muestra en la figura 2. La degradación de este compuesto se caracteriza por el desprendimiento de una molécula de propionil-CoA, en un primer ciclo de oxidación β; en el segundo ciclo, una molécula de acetil-CoA es liberada debido a la ramificación metílica presente (Pirnik *et al*., 1974).

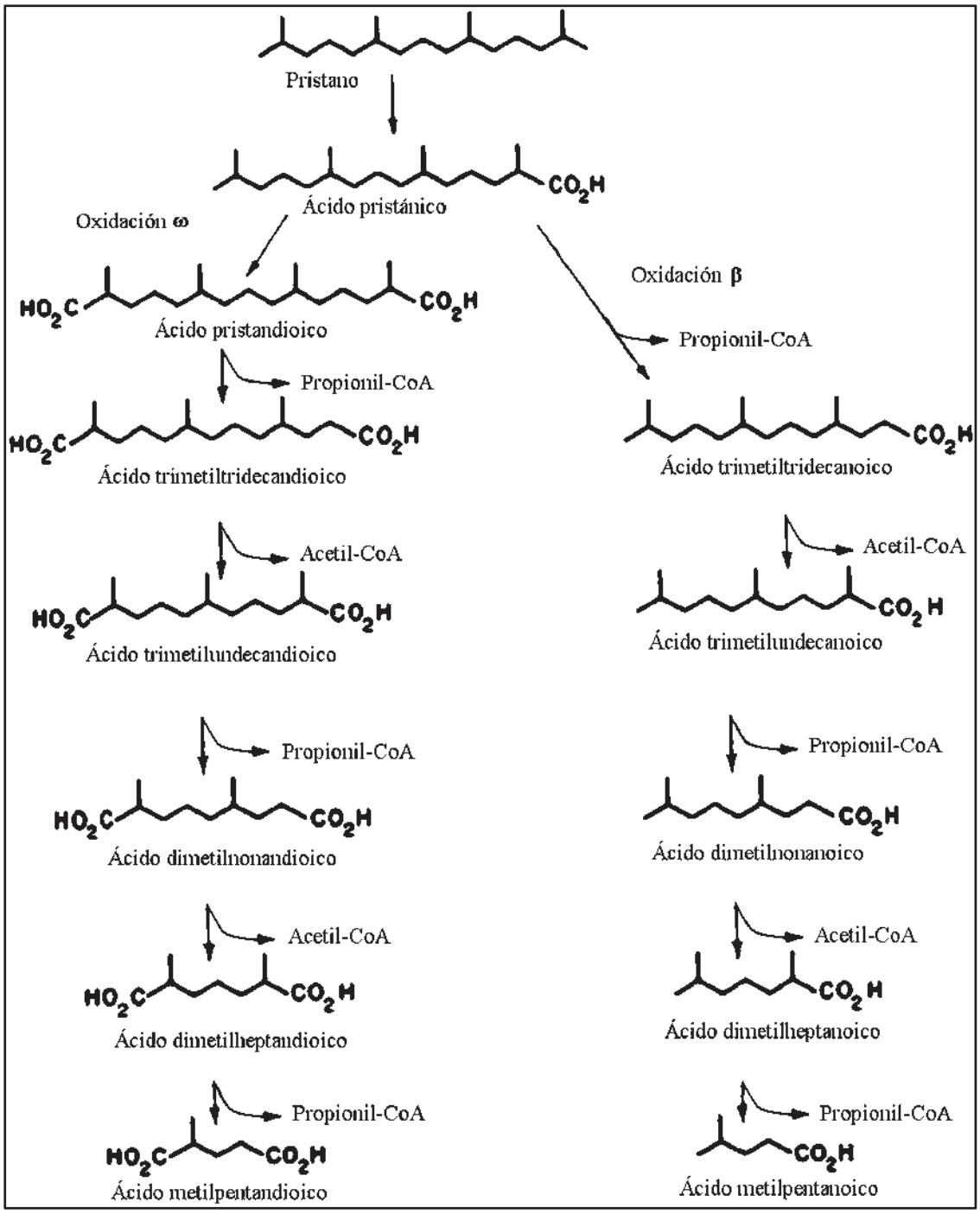


Figura 2: Ruta catabólica propuesta para la asimilación del pristano por *Brevibacterium erythrogenes.* Se inicia con la oxidación de un extremo de la cadena para formar el ácido pristánico, el cual puede sufrir la oxidación del otro extremo de la cadena (oxidación Ω), en ambos casos la degradación se da por la separación de un propionil-CoA; posterior a la siguiente reacción de oxidación se pierde una molécula de acetil-CoA (Tomado de Pirnik *et al*., 1974).

3.3. Degradación de isoprenoides/terpenos acíclicos

Los compuestos que presentan la mayor resistencia a la degradación por microorganismos son los alcanos 3-metil ramificados, que han sido descritos como compuestos ambientalmente persistentes o recalcitrantes debido a que inhiben el proceso de la oxidación β (Hector *et al.,* 1993); sin embargo, se han encontrado varias especies de microorganismos que pueden utilizar este tipo de compuestos como fuentes de carbono y energía (Cantwell *et al.,* 1978).

El catabolismo de los compuestos 3-metil ramificados es realizado por algunos organismos haciendo acopio de rutas metabólicas alternas como, la descarboximetilación β, que involucra la separación de la ramificación 3-metil por carboxilación, seguida de la remoción oxidativa del grupo carboximetilo formado para generar un sustrato susceptible de ser catabolizado por oxidación β (Scheaffer *et al.,* 1979; Hector *et al.,* 1993). Dentro de este tipo de compuestos también conocidos como β-ramificados, tenemos a los monoterpenos acíclicos de la familia del citronelol (AMTC: por sus siglas en inglés "Acyclic Monoterpenes of the Citronellol Family"), como son citronelol, geraniol y nerol. Estos son compuestos 3-metil ramificados que pueden ser asimilados por especies de la familia *Pseudomonadaceae* (*P. citronellolis*, *P. aeruginosa* y *P. mendocina*) (Cantwell *et al.,* 1978). Se ha utilizado al citronelol (3,7-dimetil-6-octen-1-ol) como molécula modelo para el estudio de la degradación de estos compuestos. El citronelol es un terpenol natural que se encuentra en las plantas del género *Citrus, Citrus limon* por ejemplo, en las que es responsable del sabor y aroma característicos de sus frutos.

3.4. Degradación de isoprenoides/terpenos acíclicos en *P. citronellolis* y *P. aeruginosa*

La ruta degradativa de isoprenoides acíclicos en *P. citronellolis* fue propuesta por Cantwell y colaboradores (1978); sin embargo, no fue hasta el 2004 que se logró relacionar un grupo de genes de *P. aeruginosa* con algunas de las actividades enzimáticas involucradas en la degradación del citronelol (Díaz-Pérez *et al.,* 2004). Estos genes se encuentran agrupados formando un operón catabólico denominado *gnyRDBHAL* (Díaz-Pérez *et al.*, 2004) y que posteriormente fue renombrado como operón *liuABCDEFGH* (Hoschle *et al.,* 2005).

La ruta general propuesta para la degradación de AMTC en *P. aeruginosa* involucra la oxidación del citronelol al ácido citronélico por medio de una serie de reacciones en las que el citronelal se presenta como intermediario (Díaz-Pérez *et al.,* 2004; Forster-Fromme y Jendrossek, 2005). Esta etapa de la ruta es denominada "superior" (Figura 3); y en ella otros AMTC estructuralmente similares al citronelol, como son el nerol y el geraniol, son transformados a geranil-CoA, el cual es el intermediario donde convergen los compuestos iniciales. En la ruta "inferior", el ácido citronélico es transformado por las enzimas: citronelil-CoA deshidrogenasa, geranil-CoA carboxilasa (GCCasa), considerada como la enzima clave de esta ruta catabólica, γ-carboxigeranil-CoA hidratasa y 3-hidroxi-γ-carboxigeranil-CoA liasa hasta acetil-CoA y 3-oxo-7-metil-octenoato. Este último es un compuesto susceptible de ser degradado por la oxidación β. Al perder cuatro átomos de carbono mediante dos ciclos de oxidación, se genera el intermediario 3-metilcrotonil-CoA, el cual puede ser metabolizado hasta acetil-CoA y acetoacetato por las enzimas que forman parte de la ruta catabólica de leucina/isovalerato.

El 3-metilcrotonil-CoA (MC-CoA) presenta una ramificación metílica en posición β (Figura 3), al igual que el geranil-CoA (G-CoA), por lo que es considerado análogo de este último, al igual que la ruta catabólica y las enzimas. Las enzimas que intervienen en la ruta degradativa de leucina/isovalerato son: 3-metilcrotonil-CoA carboxilasa (MCCasa) (enzima considerada homóloga de la GCCasa y clave de la ruta catabólica de leucina/isovalerato), 3-metilglutaconil-CoA hidratasa y 3-hidroxi-3-metilglutaril-CoA liasa (Figura 3). La ruta degradativa de leucina puede ser dividida en dos partes, la primera, consiste en la desaminación de la leucina y en posterior reacción se produce isovaleril-CoA. En la segunda etapa este metabolito es convertido a MC-CoA que, al igual que el MC-CoA proveniente de la ruta degradativa de AMTC, es transformado por las enzimas de la ruta catabólica de leucina/isovalerato hasta acetil-CoA y acetoacetato, como se puede apreciar en la figura 3 (Martin *et al.,* 1973; Díaz-Pérez *et al.,* 2004; Rodríguez *et al.,* 2004; Hoschle *et al.,* 2005).

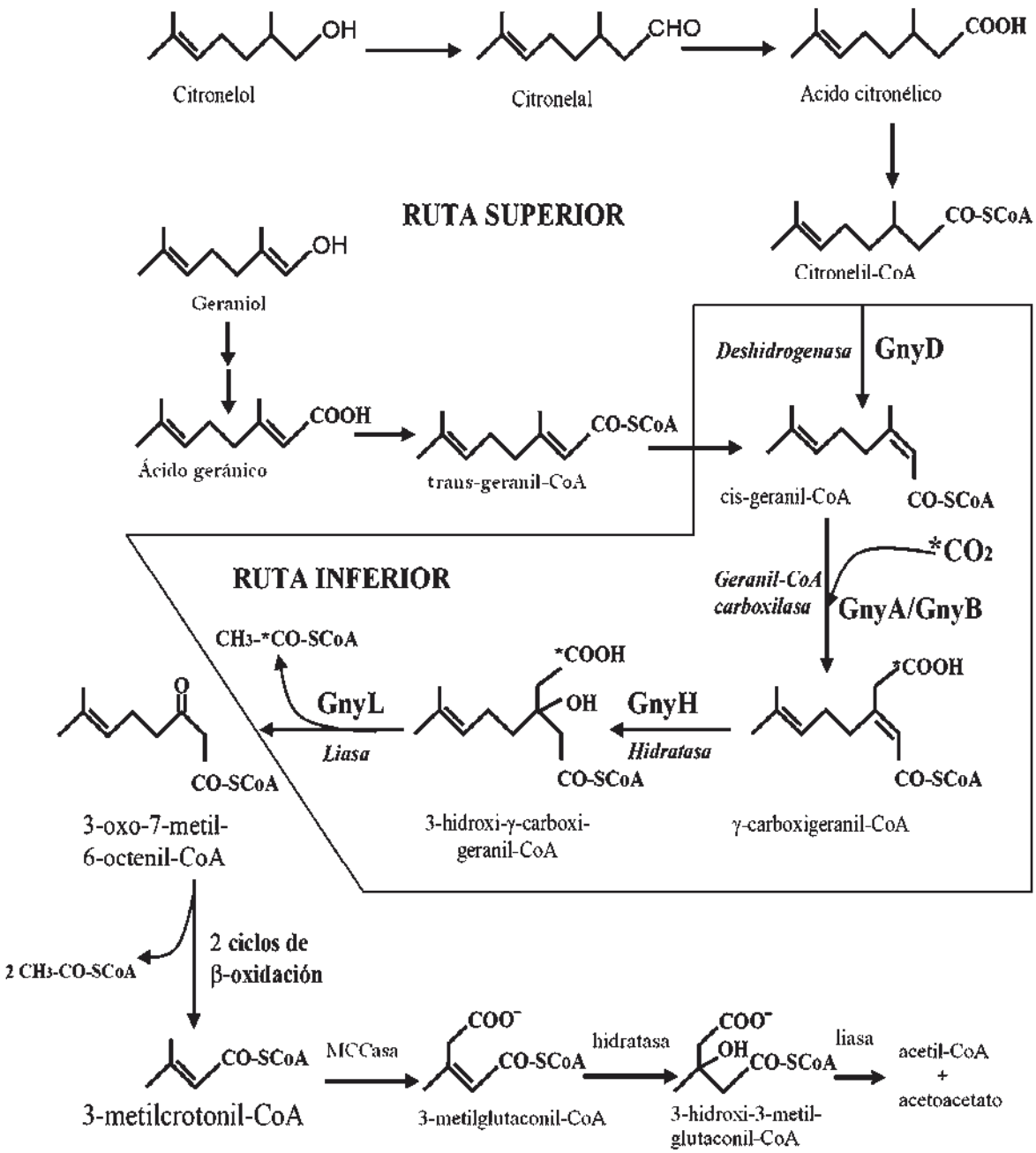


Figura 3. Ruta catabólica de AMTC propuesta para *Pseudomonas aeruginosa* PAO1; en la ruta superior se lleva a cabo la oxidación de los alcoholes citronelol y geraniol, ambos coinciden en cis-geranil-CoA que, por las enzimas de la ruta inferior, es carboxilado y hidratado, además de perder un acetil-CoA, para producir 3-oxo-7-metil-6-octenil-CoA, el cual después de dos ciclos de oxidación β, en metabolito resultante entra a la ruta degradativa de leucina para completar su degradación hasta acetil-CoA y acetoacetato (Adaptado de Díaz-Pérez *et al*., 2004).

P. citronellolis fue aislada por Seubert en 1960 por su capacidad de crecer en citronelol como única fuente de carbono y energía; el estudio de los AMTC fue originalmente iniciado en esta especie, de la cual se han purificado y caracterizado las enzimas GCCasa y MCCasa (Fall y Hector, 1977: Fall, 1981). Posteriormente, se encontró que *P. aeruginosa* también es capaz de catabolizar este compuesto y se ha sugerido que ambas especies metabolizan estos compuestos por medio de la misma ruta (Cantwell *et al*., 1978).

3.5. Enzimas dependientes de biotina

Las enzimas dependientes de biotina son un grupo de enzimas que catalizan la transferencia de un grupo carboxilo de una molécula donadora a una aceptora, en las que la biotina actúa como el transportador del grupo carboxilo. A estas enzimas, la biotina es adicionada post-traduccionalmente por la enzima biotin ligasa, en *E. coli*, es efectuado por la proteína BirA (Barker y Campbell, 1981). Estas enzimas se encuentran en todos los organismos participando en diversas rutas biosintéticas.

3.6. Estructura y función de la biotina

La biotina, también conocida como vitamina H, esta formada por un anillo bicíclico constituido por un anillo ureido y un anillo tiofano unidos a la cadena lateral de un valerato. Existen ocho estereoisómeros pero sólo uno es biológicamente activo, la D-biotina, su estructura se presenta en la figura 4A. La biotina funciona como cofactor en un conjunto de enzimas que catalizan la transferencia de un grupo carboxilo de una molécula donadora a una aceptora, actúa como transportador intermediario del grupo carboxilo a transferir. Las enzimas dependientes de biotina contienen una secuencia conservada: A(V)-M-K-M(A)-E; la biotina se encuentra unida al amino épsilon (ε) del residuo de lisina por medio de un enlace covalente. Cuando una molécula de CO_2 se encuentra unida a la biotina, se le conoce como carboxi-biotina (figura 4B) (Jitrapakdee y Wallace, 2003). Las enzimas dependientes de biotina están involucradas en procesos metabólicos importantes como son: la lipogénesis, la gluconeogénesis y el metabolismo de aminoácidos (Samols *et al.,* 1988).

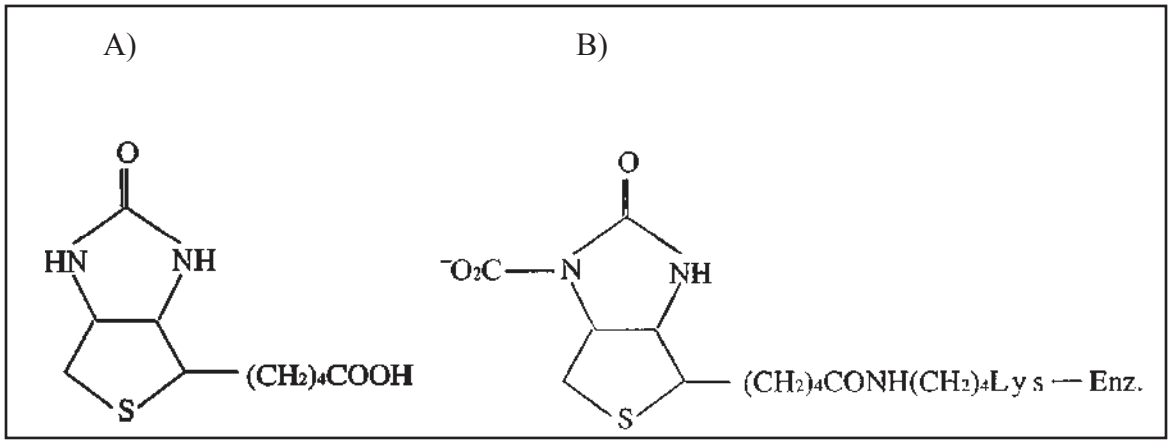


Figura 4. Estructura de la biotina. A) Modelo de la D-biotina. B) Esquema que muestra la unión de la carboxi-biotina al residuo de lisina de la enzima.

La biotina se une fuertemente a la avidina y estreptavidina. La avidina es una glicoproteína presente en la clara de los huevos de algunas aves, mientras que su contraparte bacteriana, la estreptavidina, se extrae de *Streptomyces avidinii*. Ambos compuestos tienen propiedades similares la principal es la gran afinidad que tienen por la biotina, a la que se unen a través de enlaces no covalentes con una constante de disociación de $\sim 10^{-15}$ M^{-1}. Esto permite purificar enzimas que contengan biotina como grupo prostético, utilizando cromatografía de afinidad y ser detectadas por medio de Western blot (Jitrapakdee y Wallace, 2003).

3.7. Enzimas con biotina como grupo prostético

Las enzimas dependientes de biotina se clasifican en tres clases de acuerdo a su especificidad de sustrato:

Clase I: También conocidas como carboxilasas, en esta clase se encuentran las enzimas que utilizan al bicarbonato como molécula donadora del carboxilo y son dependientes de ATP y Mg^{2+}; entre ellas se encuentran las enzimas acetil-CoA, propionil-CoA, 3-metilcrotonil-CoA, geranil-CoA, urea y piruvato carboxilasas.

Clase II: A esta clase pertenecen las enzimas descarboxilasas, las cuales catalizan la descarboxilación de algunos ácidos orgánicos; no son dependientes de ATP. Aquí se incluyen la oxaloacetato, metilmalonil y glutacolil descarboxilasas.

Clase III: Conocidas como transcarboxilasas. La enzima transcarboxilasa purificada de *Propionibacterium shermanii* es la única enzima de esta clase. La transcarboxylasa realiza la transferencia de un grupo carboxilo del metilmalonil-CoA y lo adiciona a una molécula de piruvato, de esta manera produce propionil-CoA y oxaloacetato (Wood y Barden, 1977; Hall *et al*., 2003).

3.8. Estructura de enzimas dependientes de biotina

Para que una enzima dependiente de biotina realice la carboxilación requiere de tres actividades para la catálisis de la reacción: 1) actividad de biotin carboxilasa, corresponde a la primera mitad de la reacción (Figura 5A); 2) actividad de carboxiltransferasa, cataliza la segunda mitad de la reacción, (Figura 5B); y 3) transferencia de carboxi-biotina, que une físicamente a las dos primeras funciones (Climent y Rubio, 1986).

A) $HCO_3^- + Mg\text{-}ATP + enzima\text{-}biotina \leftrightarrow enzima\text{-}biotina\text{-}CO_2^- + Mg\text{-}ADP + Pi$

B) $Enzima\text{-}biotina\text{-}CO_2^- + acil\text{-}CoA \leftrightarrow enzima\text{-}biotina + acil\text{-}CO_2\text{-}CoA$

Figura 5: Reacciones químicas efectuadas por las carboxilasas clase I. En la primera parte de la reacción (A) el ATP y el bicarbonato, que funciona como donador del CO_2^-, se unen a la enzima para formar carboxibiotina y ADP y Pi como los primeros dos productos. En la segunda parte (B) el complejo carboxibiotina-enzima reacciona con un acil-CoA para anexar el grupo carboxilo al acil-CoA y dejar libre a la biotina-enzima.

La organización de las subunidades de las enzimas dependientes de biotina es en base al número de cadenas polipeptídicas en las que se encuentran localizados los dominios funcionales. Existen enzimas que contienen las tres funciones en subunidades separadas, por ejemplo: la acetil-CoA carboxilasa (ACCasa) de *E. coli*, la oxaloacetato descarboxilasa (Dimroth *et al.,* 1970) y la transcarboxilasa de *Propionibacterium shermanii* (Wood y Kumar, 1985). De las tres subunidades, solo una es biotinilada (Figura 6A). Un caso aparte lo forma la metilmalonil–CoA descarboxilasa que está formada de cuatro subunidades y solo una es biotinilada (Hilpert y Dimroth, 1983). Otro tipo de enzimas biotina-dependientes son las que contienen a los tres dominios funcionales en dos polipéptidos distintos, por ejemplo: la MCCasa de animales (Lau *et al.,* 1979) y bacteriana (Figura 6B) (Fall y Hector, 1977); y la GCCasa de *P. citronellolis* (Hector y Fall, 1976). Otro caso lo presentan las enzimas en las que las tres funciones están presentes en un solo polipéptido; por ejemplo, la ACCasa de pollo (Figura 6C) (Takai *et al*., 1988), plantas (Egli *et al*., 1993) y levadura (Al-Feel *et al*., 1992).

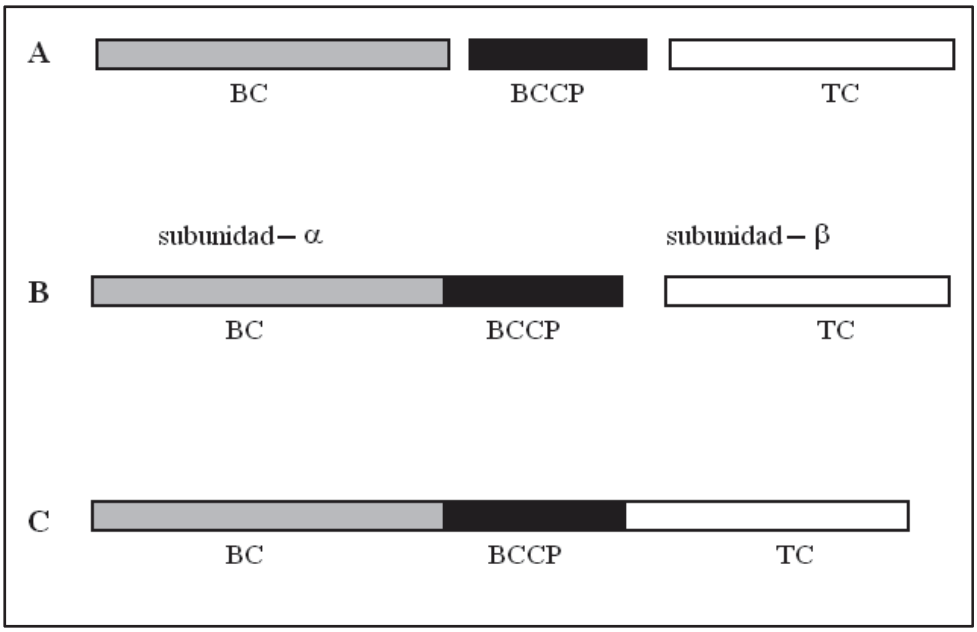


Figura 6. Representación esquemática de los diferentes tipos de carboxilasas biotina-dependientes, de acuerdo al arreglo de los dominios en la cadena polipeptídica. A) Arreglo de la acetil-CoA carboxilasa de *E. coli*, que muestra los tres dominios en diferentes cadenas polipeptídicas; B) Dos polipéptidos (subunidades α y β) forman a la MCCasa bacteriana; en el primero se encuentran localizados dos dominios; C) Cadena polipeptídica en la que se localizan los tres dominios de la acetil-CoA carboxilasa de pollo. BC: dominio biotin-carboxilasa; BCCP: dominio acarreador de biotin-carboxilo; TC: dominio carboxiltransferasa. (Adaptada de Jitrapakdee y Wallace, 2003).

3. 9. Función de la 3-metilcrotonil-CoA carboxilasa en la degradación de leucina

La enzima 3-metilcrotonil-CoA carboxilasa (MCCasa, E.C.6.4.1.4) cataliza la carboxilación del 3-metilcrotonil-CoA (MC-CoA) para formar 3-metilglutaconil-CoA (MG-CoA) en una reacción dependiente de ATP y Mg^{2+}. Este ha sido considerado como un paso de suma importancia en la ruta catabólica del aminoácido leucina, así como de isoprenoides y del mevalonato (Wurtele y Nikolau, 2000). La MCCasa está conformada por dos subunidades, una de las cuales es biotinilada (α) y una libre de biotina (β). En eucariontes se observó que la MCCasa presenta una estructura cuaternaria $\alpha_6\beta_6$ (Lau *et al*., 1980), mientras que en *P.*

citronellolis es $\alpha_4\beta_4$ (Fall y Hector, 1977; Fall, 1981); en papa se ha observado que su estructura es $\alpha_4\beta_4$ (Wurtele y Nikolau, 2000).

El mecanismo cinético de la enzima MCCasa de plantas sigue un mecanismo al azar Bi-Bi Uni-Uni Ping-Pong (Finlayson y Dennis, 1983; Knowles, 1989; Wurtele y Nikolau, 2003), como se muestra en la figura 7.

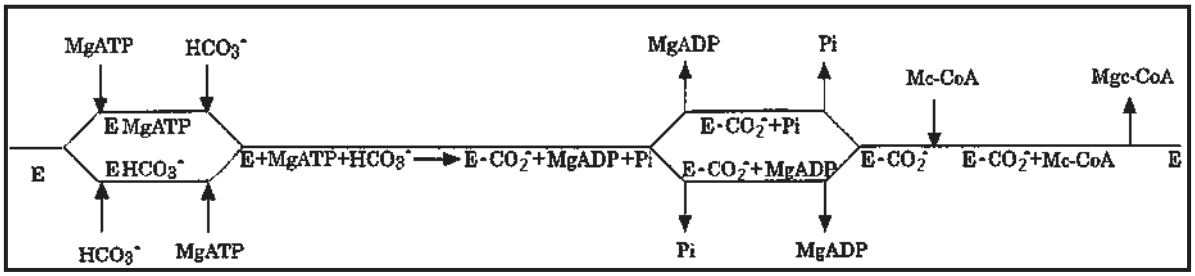


Figura 7: Representación esquemática del mecanismo cinético, al azar bi-bi uni-uni ping-pong de la MCCasa de plantas. La enzima (E) puede unirse primero al MgATP o al bicarbonato, a continuación se une al compuesto faltante para efectuar la reacción. Del mismo modo, al liberar los productos, puede liberar primero al MgADP o al pirofosfato (Pi); una vez liberados ambos compuestos y formado el intermediario E-CO2-, se une el aceptor del carboxilo (Mc-CoA) una vez realizada la reacción, el producto (Mgc-CoA) es liberado y la enzima (E) es liberada (Tomado de Wurtele y Nikolau, 2000).

Se ha descrito que algunas MCCasas, además de carboxilar al MC-CoA, también carboxilan al crotonil-CoA aunque con una velocidad mucho menor; en otros casos, las MCCasas bacteriana y animal pueden carboxilar acetoacetil-CoA, mientras que para la enzima proveniente de plantas este compuesto es un fuerte inhibidor (Wurtele y Nikolau, 2000).

Recientemente se encontró que en *P. aeruginosa* crecida en leucina o en citronelol como única fuente de carbono esta enzima es inducida; además, fueron identificados los genes que podrían codificar las subunidades que conforman a esta enzima, así como otras enzimas

que pudieran estar involucradas en los pasos siguientes del catabolismo de la leucina (Díaz-Pérez *et al.,* 2004; Hoschle *et al.,* 2005). Sin embargo, la función de estas proteínas no se ha comprobado.

3.10. Función de la geranil-CoA carboxilasa en la degradación de AMTC

La enzima geranil-CoA carboxilasa (GCCasa, E.C.6.4.1.5) ha sido estudiada en *P. citronellolis* (Hector y Fall, 1976) y en plantas (Guan *et al*., 1999). En ambos casos, se encontró que está involucrada en la degradación de isoprenoides acíclicos. En bacterias, esta enzima es inducible cuando el microorganismo es crecido en citronelol, ácido citronélico o ácido geránico como única fuente de carbono y energía. Es considerada homóloga, tanto en actividad como en estructura, a la MCCasa además de que se ha observado que en *P. citronellolis* estas dos enzimas presentan propiedades similares (Fall y Hector, 1977).

En *P. aeruginosa*, la GCCasa es inducida al crecer el microorganismo en citronelol como única fuente de carbono, identificando los probables genes que codifican las subunidades α y β de esta enzima (Díaz-Pérez *et al.*, 2004; Hoschle *et al.,* 2005); sin embargo, aún se conoce poco sobre las propiedades bioquímicas de ésta.

3.11. Controversia sobre la bifuncionalidad de las enzimas MCCasa y GCCasa

La mayoría de las enzimas son específicas para un sólo sustrato; sin embargo, algunas enzimas tienen afinidad por dos sustratos o más. En el caso específico de las carboxilasas, se han encontrado algunas que pueden carboxilar dos o más compuestos; en estos casos se les ha denominado acil-CoA carboxilasas. Un ejemplo lo presentan la acetil-CoA carboxilasa y la propionil-CoA carboxilasas de *Streptomyces coelicolor*, que además de actuar sobre su sustrato, también pueden hacerlo sobre el sustrato de la otra y pueden carboxilar al butiril-CoA (Diacovich *et al*., 2002).

Se ha sugerido que la MCCasa, así como su homóloga, la GCCasa provenientes de *P. citronellolis,* pueden carboxilar al sustrato de la otra debido a la similitud estructural entre ambos compuestos; sin embargo, se ha observado que la MCCasa muestra cierta especificidad de sustrato, mientras que la GCCasa puede actuar sobre otros acil-CoA, incluyendo al MC-

CoA (Fall, 1981; Guan *et al*., 1999). No obstante, aún existe controversia en la bifuncionalidad de alguna de las enzimas en las rutas degradativas de citronelol y de leucina, ya que en algunos casos, para la determinación de la actividad enzimática de la GCCasa y la MCCasa se han utilizado reacciones acopladas con la piruvato cinasa y la lactato deshidrogenasa. Forster-Fromme y colaboradores (2006) sugieren que la GCCasa y la MCCasa de *P. aeruginosa* realizan su actividad enzimática de una manera específica por el sustrato. Por otro lado, datos obtenidos por nuestro grupo utilizando herramientas genéticas, sugieren que ambas rutas catabólicas pueden compartir productos génicos o alguna enzima pudiera funcionar en ambas rutas mostrando bifuncionalidad (Díaz-Pérez *et al.,* 2004).

IV. ANTECEDENTES

Pseudomonas aeruginosa es una bacteria gran negativa considerada ubicua ya que presenta una gran versatilidad metabólica que le permite crecer en una gran variedad de compuestos orgánicos, entre los que se encuentra los alcanos de cadenas ramificadas. Cantwell y colaboradores (1978), propusieron la ruta degradativa de alcanos β ramificados (isoprenoides acíclicos, concretamente) utilizada por *P. citronelollis*. El citronelol es el isoprenoide acíclico (AMTC: Acyclic monoterpenes type citronelol) que se ha utilizado como modelo para el estudio del catabolismo de los compuestos con estructura β ramificada. *P. aeruginosa* PAO1 también es capaz de utilizarlo como fuente de carbono y energía. En el laboratorio se identificaron dos grupos de genes en el cromosoma de *P. aeruginosa* PAO1 involucrados en la degradación de citronelol (isoprenoides acíclicos) y de leucina/isovalerato, el primero fue designado como operón *atu* (acyclic terpene utilization) y está conformado por los genes *atuABCDEFG* y el segundo como operón *liu* (leucine-isovalerate utilization) formado por los genes *liuRABCD* (*gnyRDBHAL*) (Díaz-Pérez 2003; Hoschle *et al.,* 2005), son operones homólogos ya que las funciones enzimáticas codificadas son similares. Al realizar una comparación de secuencias, los péptidos AtuCF y LiuBD presentaron homología con las subunidades β y α de enzimas acil-CoA carboxilasas, geranil-CoA carboxilasa y 3-metilcrotonil-CoA carboxilasa, respectivamente. La 3-metilcrotonil-CoA carboxilasa se sugiere que participa en la conversión de 3-metilcrotonil-CoA en 3-metilglutaconil-CoA de la ruta catabólica de leucina; y la geranil-CoA carboxilasa en la conversión de geranil-CoA en γ-carboxigeranil-CoA de la ruta catabólica de isoprenoides acíclicos. Ambas enzimas de *P. citronellolis* han sido caracterizadas, así como la 3-metilcrotonil-CoA carboxilasa de otros organismos procariontes y eucariontes. Se ha observado que las enzimas carboxilasas catalizan los pasos limitantes de las rutas metabólicas en las que participan, por lo que se ha considerado que son las enzimas clave. Asimismo, se han obtenido datos que sugieren que las enzimas geranil-CoA y 3-metilcrotonil-CoA carboxilasas pudieran estar interviniendo en las dos rutas catabólicas, contrario a lo que se había reportado para las enzimas de *P. citronellolis* (Fall y Hector, 1977; Hoschle *et al.,* 2005). Por lo anterior, se decidió comenzar el análisis de las enzimas de estas rutas metabólicas, con la caracterización de las dos enzimas carboxilasas.

V. HIPOTESIS

Los genes *atuC/atuF* y *liuB/liuD* de *Pseudomonas aeruginosa* codifican a las enzimas geranil-CoA carboxilasa (GCCasa) y 3-metilcrotonil-CoA carboxilasa (MCCasa), que se encuentran involucrados en el catabolismo de isoprenoides acíclicos y de leucina, respectivamente.

VI. OBJETIVOS

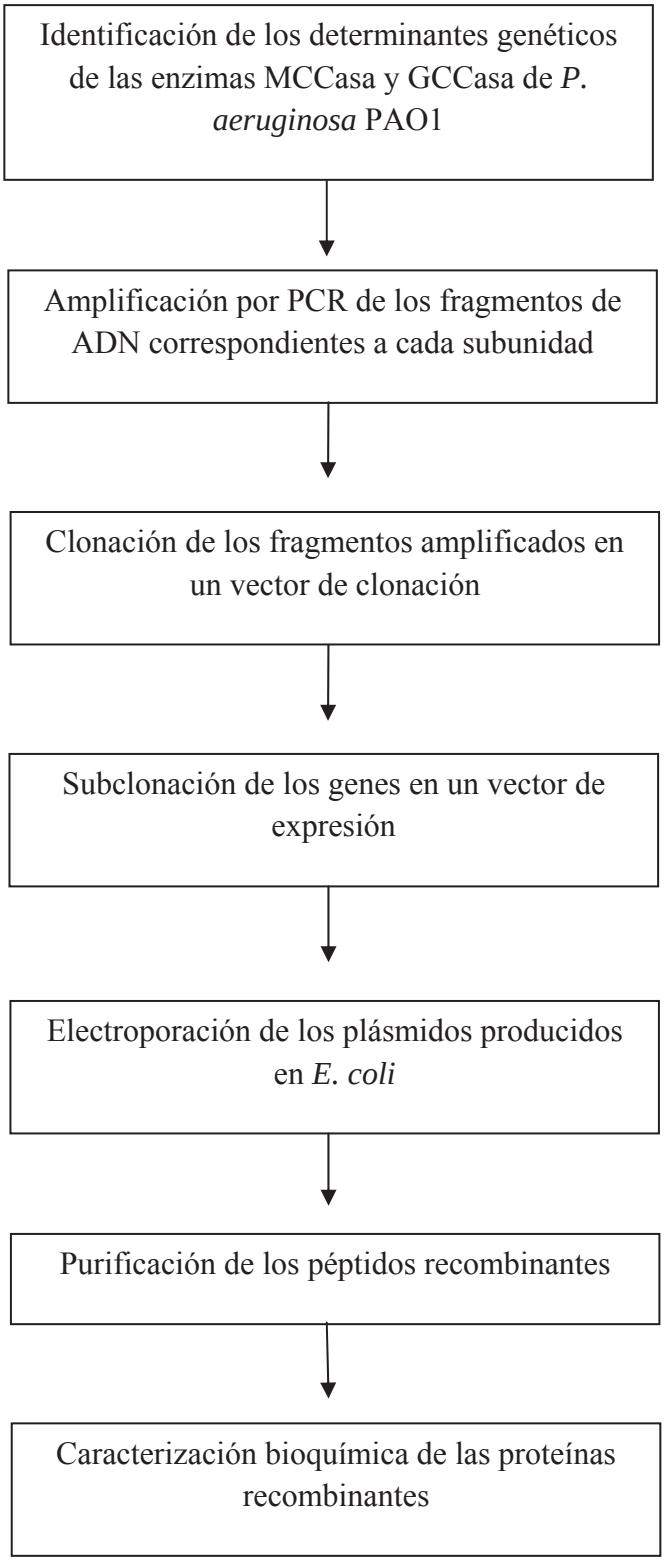
OBJETIVO GENERAL:

Caracterizar los determinantes genéticos codificantes de las enzimas geranil-CoA carboxilasa y 3-metilcrotonil-CoA carboxilasa, involucradas en el catabolismo de isoprenoides acíclicos y leucina en *Pseudomonas aeruginosa* PAO1.

OBJETIVOS ESPECIFICOS:

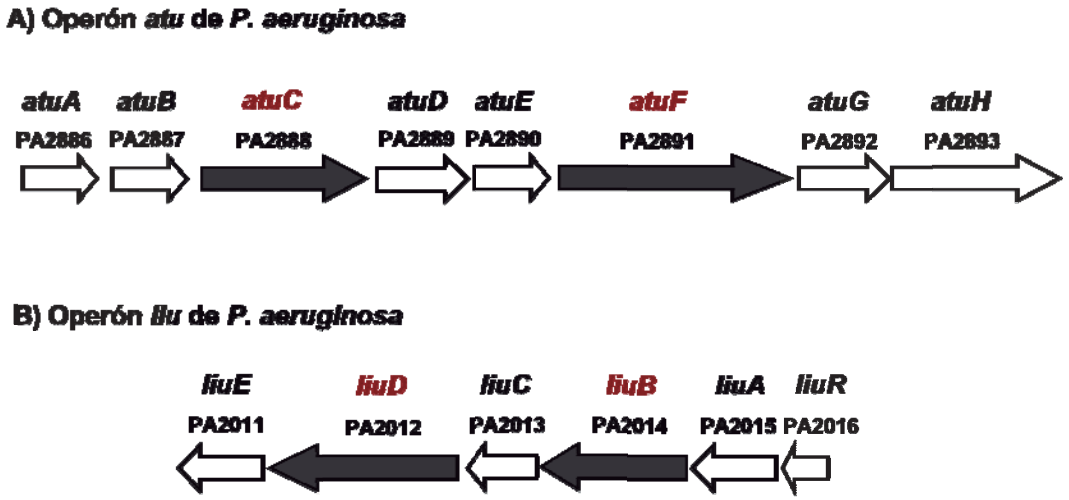
1. Identificar los determinantes genéticos involucrados en el catabolismo de isoprenoides acíclicos y leucina en *Pseudomonas aeruginosa*.
2. Expresar, purificar y caracterizar cinéticamente las enzimas geranil-CoA carboxilasa y 3-metilcrotonil-CoA carboxilasa de *P. aeruginosa*.

VII. ESTRATEGIA METODOLOGIA



1. Identificación de los determinantes genéticos de las enzimas MCCasa y GCCasa de *P. aeruginosa* PAO1.

   Los marcos de lectura abiertos en el genoma de *P. aeruginosa* PAO1 identificados, que codifican para las subunidades α y β de las enzimas GCCasa y MCCasa son: PA2891 (*atuF*), PA2888 (*atuC*), PA2012 (*liuD*) y PA2014 (*liuB*); respectivamente (www.pseudomonas.com).



2. Amplificación por PCR de los fragmentos de ADN correspondientes a cada subunidad.

   En base a la secuencia reportada para estos genes, se diseñaron oligonucleótidos que insertan sitios de reconocimiento para enzimas de restricción:

   liuD:

   5’-A GGATCC [a] CATGAACCCGGACTACCGCAGCATC-3’
   5’-G AAGCTT [b] GGCCTGGTTCTCGTCCAGCTC-3’

   liuB:

   5’-C GGATCC [a] CATGGCCATCCTGCACACCCAGATC-3’
   5’-C AAGCTT [b] CATGCGGAACACGCCGAAGGC-3’

atuF:

5’-GGATCC [a] CATGCCCAGCTTCAACAAGA-3’
5’-AAGCTT [b] CGTCGGCTTCCACCTCGACC-3’

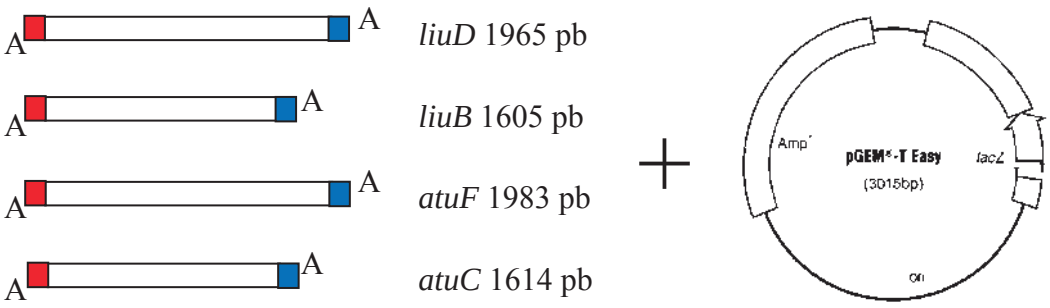
atuC:

5’-A GGATCC [a] CATGCCCGCGATCCAGTCGG-3’
5’-G AAGCTT [b] CCCTCGCGACCCCGAAGCTG-3’

[a] Región de reconocimiento de la enzima *Bam*H I
[b] Región de reconocimiento de la enzima *Hin*d III

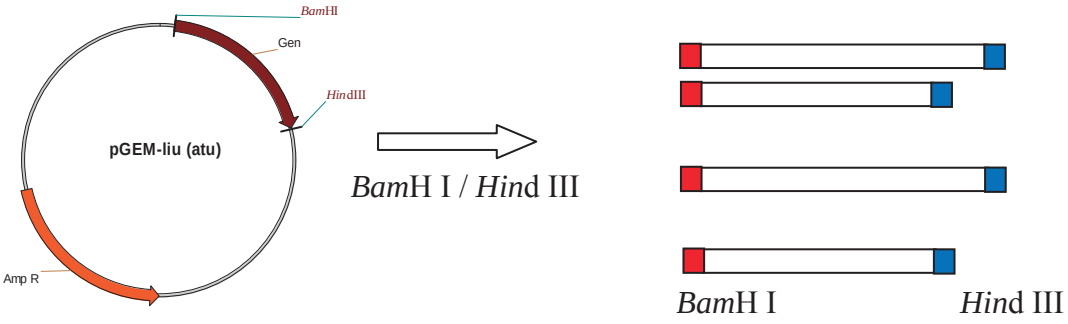
3. Clonación de los fragmentos amplificados en un vector de clonación.

El producto de la reacción de PCR fue ligado al vector de clonación pGEM-T Easy, y los plásmidos obtenidos fueron transformados en *E. coli*.

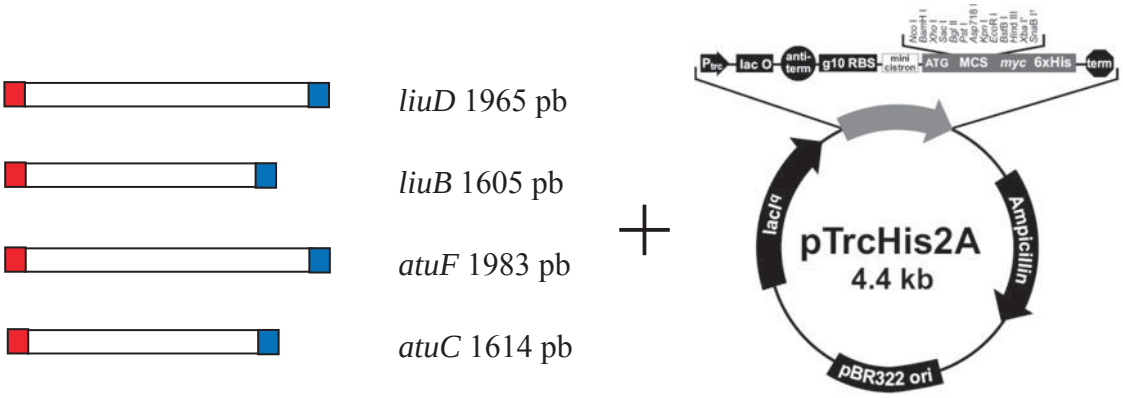


4. Subclonación de los genes en un vector de expresión.

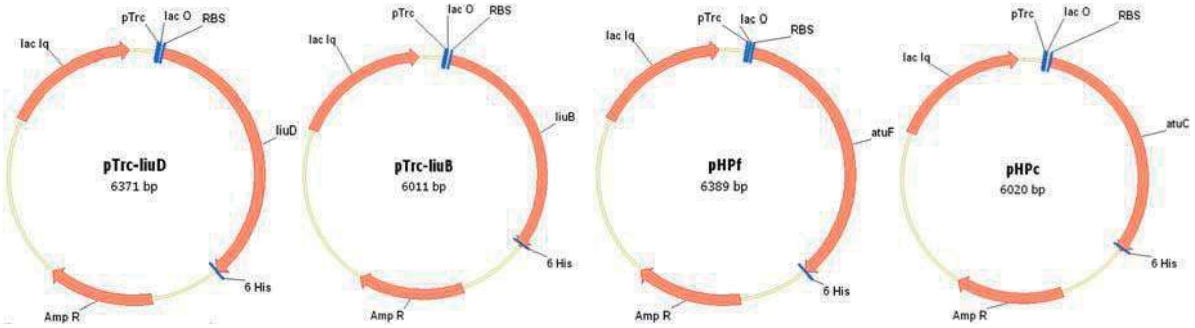
Los plásmidos con los genes fueron digeridos con las enzimas de restricción *Bam*H I y *Hin*d III.



Los fragmentos obtenidos, correspondientes al tamaño de los genes de interés, fueron clonados en el vector de expresión pTrcHis 2A.



Creandose las siguientes construcciones:



5. Electroporación de los plásmidos producidos en *E. coli.*

   Las construcciones elaboradas fueron electroporadas en células competentes de E. coli TOP10.

6. Purificación de los péptidos recombinantes.

   La purificación se realizó a partir de extracto crudo del cultivo crecido durante ~4 horas, en medio liquido, después de la inducción con IPTG. Se utilizó resina de sefarosa con cobalto, en un proceso de batch.

7. Caracterización bioquímica de las proteínas recombinantes.

   La caracterización se efectúo de acuerdo al método de fijación de radiactividad en compuestos solubles resistentes al ácido, el cual se describe en el segundo artículo en la sección de resultados.

VIII. RESULTADOS

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The *atu* and *liu* Clusters Are Involved in the Catabolic Pathways for Acyclic Monoterpenes and Leucine in *Pseudomonas aeruginosa*†

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Evidence suggests that the *Pseudomonas aeruginosa* PAO1 *gnyRDBHAL* cluster, which is involved in acyclic isoprenoid degradation (A. L. Díaz-Pérez, N. A. Zavala-Hernández, C. Cervantes, and J. Campos-García, Appl. Environ. Microbiol. 70:5102–5110, 2004), corresponds to the *liuRABCDE* cluster (B. Hoschle, V. Gnau, and D. Jendrossek, Microbiology 151:3649–3656, 2005). A *liu* (leucine and isovalerate utilization) homolog cluster was found in the PAO1 genome and is related to the catabolism of acyclic monoterpenes of the citronellol family (AMTC); it was named the *atu* cluster (acyclic terpene utilization), consisting of the *atuCDEF* genes and lacking the hydroxymethyl-glutaryl-coenzyme A (CoA) lyase (HMG-CoA lyase) homolog. Mutagenesis of the *atu* and *liu* clusters showed that both are involved in AMTC and leucine catabolism by encoding the enzymes related to the geranyl-CoA and the 3-methylcrotonyl-CoA pathways, respectively. Intermediary metabolites of the acyclic monoterpene pathway, citronellic and geranic acids, were accumulated, and leucine degradation rates were affected in both *atuF* and *liuD* mutants. The alpha subunit of geranyl-CoA carboxylase and the alpha subunit of 3-methylcrotonyl-CoA carboxylase (α-MCCase), encoded by the *atuF* and *liuD* genes, respectively, were both induced by citronellol, whereas only the α-MCCase subunit was induced by leucine. Both citronellol and leucine also induced a LacZ transcriptional fusion at the *liuB* gene. The *liuE* gene encodes a probable hydroxy-acyl-CoA lyase (probably HMG-CoA lyase), an enzyme with bifunctional activity that is essential for both AMTC and leucine degradation. *P. aeruginosa* PAO1 products encoded by the *liuABCD* cluster showed a higher sequence similarity (77.2 to 79.5%) with the probable products of *liu* clusters from several *Pseudomonas* species than with the *atuCDEF* cluster from PAO1 (41.5%). Phylogenetic studies suggest that the *atu* cluster from *P. aeruginosa* could be the result of horizontal transfer from *Alphaproteobacteria*. Our results suggest that the *atu* and *liu* clusters are bifunctional operons involved in both the AMTC and leucine catabolic pathways.

The 3-methyl branched alkanes (3-MBA) have been described as environmentally persistent compounds, although several species of microorganisms can use 3-MBA as the sole carbon source (28). Acyclic monoterpenes of the citronellol family (AMTC) are 3-MBA compounds that can be used by the *Pseudomonadaceae* (*Pseudomonas citronellolis*, *Pseudomonas aeruginosa*, and *Pseudomonas mendocina*) (2, 3). The pathways for the degradation of AMTC in *P. citronellolis* (3) and plants (9) have been reported, but only for *P. aeruginosa* have the genes involved been identified (5, 12). The proposed general route for citronellol degradation in *P. aeruginosa* first involves the oxidation of the alcohol to citronellic acid (called the upper pathway). In the lower pathway, citronellic acid is transformed to acetyl coenzyme A (acetyl-CoA) and 3-oxo-7-methyl-octenoate, a suitable substrate for the β-oxidation route. These transformations are suggested to be carried out by the enzymes citronellyl-CoA dehydrogenase, geranyl-CoA carboxylase (GCCase), γ-carboxygeranyl-CoA hydratase, and 3-hydroxy-γ-carboxygeranyl-CoA lyase (5, 12). After two rounds of β-oxidation, 3-methylcrotonyl-CoA (MC-CoA) is generated (Fig. 1). This metabolite may be transformed to acetyl-CoA and acetoacetate by the 3-methylcrotonyl-CoA carboxylase (MCCase), which is involved in leucine and AMTC catabolic pathways, as has been shown to occur in bacteria (5, 6, 12), fungi (26), and plants (1, 9, 20). Two pathways have been proposed for leucine catabolic breakdown in microbes; the most common involves the MC-CoA pathway to produce acetyl-CoA and acetoacetate (22). In *P. aeruginosa*, *Pseudomonas putida*, and *Pseudomonas fluorescens*, leucine catabolism occurs by the MC-CoA pathway (4, 12, 17, 18, 19). Leucine transaminase first converts leucine to 2-ketoisocaproic acid, and 2-ketoisocaproic dehydrogenase renders isovaleryl-CoA. This metabolite is converted to 3-methylcrotonyl-CoA by isovaleryl-CoA dehydrogenase; 3-methylcrotonyl-CoA carboxylase then produces 3-methylglutaconyl-CoA, which is converted to 3-hydroxy-3-methylglutaryl-CoA by 3-methylglutaconyl-CoA hydratase; and finally acetyl-CoA and acetoacetate are produced by 3-hydroxy-3-methylglutaryl-CoA lyase (12, 17, 18, 19, 26) (Fig. 1).

We previously described the *P. aeruginosa gnyRDBHAL* gene cluster, which encodes the enzymes for the AMTC degradation lower pathway (5); in a recent paper this was renamed the *liuRABCDE cluster* (12). Following our study (5), a highly conserved homologous cluster corresponding to the *atu* cluster was identified (12). In this study, we characterized the *atu* cluster, and we propose that the *atu* and *liu* clusters encode the enzymes involved in both AMTC and leucine utilization; the evolutionary relation of these gene clusters is also analyzed.

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† Supplemental material for this article may be found at http://aem.asm.org/.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this work are shown in Table 1. The strains were grown at 30°C in

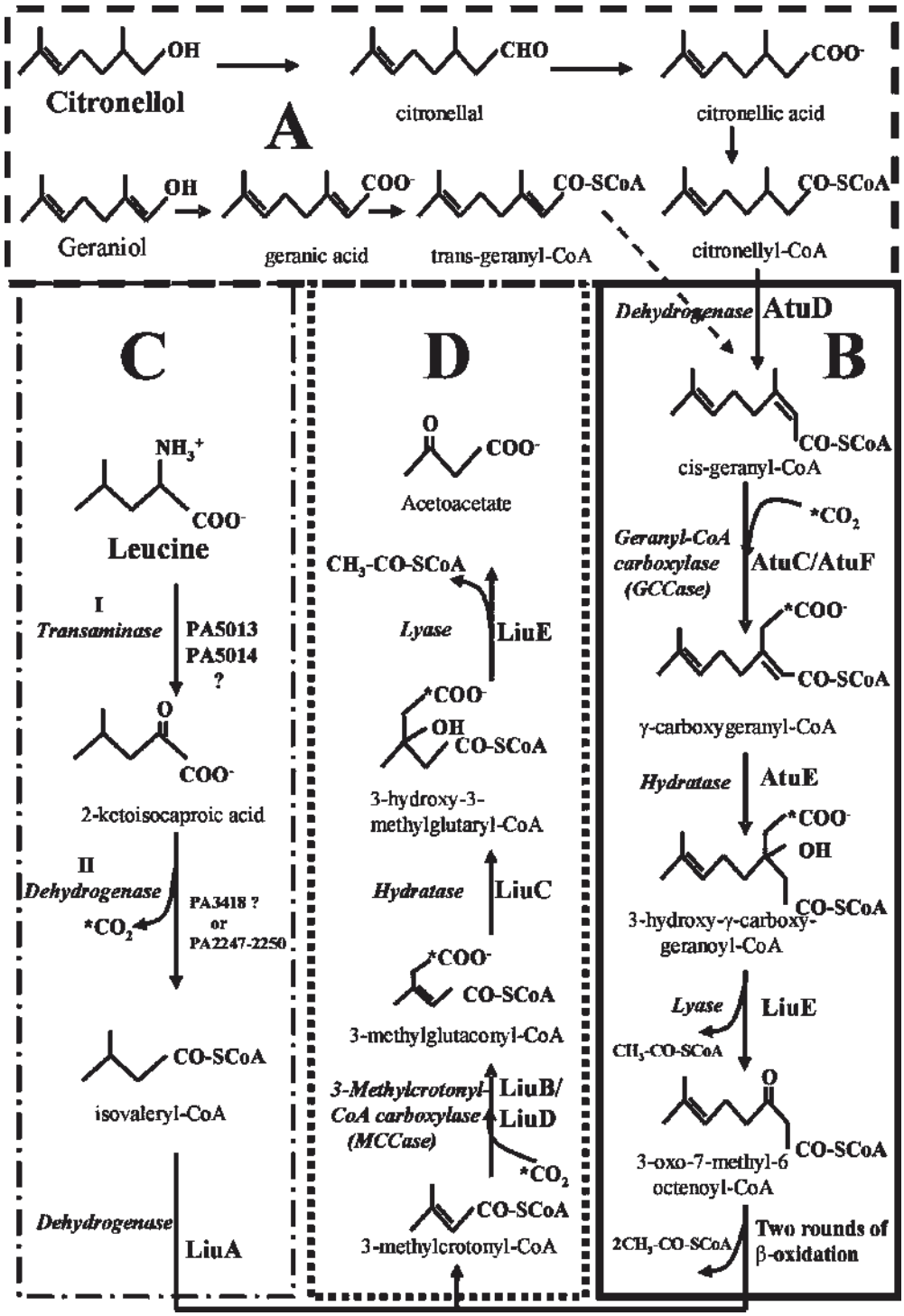

FIG. 1. Catabolic pathways and proposed functions of the *atu* and *liu* cluster products from *P. aeruginosa* PAO1. (A and B) Upper and lower pathways of acyclic monoterpene catabolism; (C and D) Upper and lower pathways of leucine catabolism. AtuD, citronellyl-CoA dehydrogenase; AtuC/AtuF, geranyl-CoA carboxylase; AtuE, γ-carboxygeranyl-CoA hydratase; LiuE, 3-hydroxy-γ-carboxygeranyl-CoA and 3-hydroxy-3-methylglutaryl-CoA lyase; I, leucine transaminase; II, 2-ketoisocaproic dehydrogenase; LiuA, isovaleryl-CoA dehydrogenase; LiuB/LiuD, 3-methylcrotonyl-CoA carboxylase; LiuC, 3-methylglutaconyl-CoA hydratase. (Adapted from references 5, 9, 12, and 26.)

Luria broth or in M9 minimal medium (27). Solid media were prepared by adding 1.5% agar. Strains were tested for their ability to grow on M9 agar plates supplemented with citronellol (Merck) as the sole carbon and energy source (added as vapor) after an incubation for 48 h at 30°C. The growth of strains on branched-chain amino acids was tested as described by Martin et al. (18), using 0.3% (wt/vol) L-leucine or L-isoleucine supplemented with L-valine and L-isoleucine at 0.005% each (obtained from Sigma and Merck Co.). Antibiotic concentrations used for *P. aeruginosa* strains were as follows: streptomycin, 200 μg ml^{-1};

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
P. aeruginosa PAO1SM	Spontaneous streptomycin-resistant mutant derived from strain PAO1	33
P. fluorescens L1	Environmental strain	Donated by E. Valencia
P. putida	Environmental strain	Donated by S. Aguilera
PAO *atuC*	PAO1::IS*lac*Z/hah (ID 10287), transposon insertion in ORF PA2888	13
PAO *atuD*	PAO1::IS*lac*Z/hah (ID 15357), transposon insertion in ORF PA2889	13
PAO *atuE*	PAO1::IS*lac*Z/hah (ID 8388), transposon insertion in ORF PA2890	13
PAO *atuF*	PAO1::IS*lac*Z/hah (ID 10788), transposon insertion in ORF PA2891	13
PAO2893	PAO1::IS*lac*Z/hah (ID 8377), transposon insertion in ORF PA2893	13
PAO *liuD*	PAO1::IS*lac*Z/hah (ID 11254), transposon insertion in ORF PA2012	13
PAM1529 (*atuF*)	PAO1SM mutant obtained by Himar1::Gm^r transposition in ORF PA2891, unable to grow on citronellol	This work
PAE80 (*liuD*)	PAO1SM mutant obtained by Himar1::Gm^r transposition in ORF PA2012, unable to grow on citronellol	5
PAO *atuF liuD*	Double mutant strain derived from PAO *atuF*, mutated in *liuD* gene	This work
PAO *atuF liuB*	Double mutant strain derived from PAO *atuF*, mutated in *liuB* gene	This work
PAO *atuF liuE*	Double mutant strain derived from PAO *atuF*, mutated in *liuE* gene	This work
PAO *atuC liuE*	Double mutant strain derived from PAO *atuC*, mutated in *liuE* gene	This work
PAM *liuA*	PAO1SM *liuA*::Gm^r mutant obtained by recombination in ORF PA2015	5
PAM *liuB*	PAO1SM *liuB*::Gm^r mutant obtained by recombination in ORF PA2014	5
PAM *liuD*	PAO1SM *liuD*::Gm^r mutant obtained by recombination in ORF PA2012	5
PAM *liuE*	PAO1SM *liuE*::Gm^r mutant obtained by recombination in ORF PA2011	5
Plasmids		
pUCP20	pUC19-derived *E. coli-Pseudomonas* shuttle vector; Ap^r Cb^r	32
pMO013850	pLA2917 cosmid plus 25 kb of PAO1 genome, including the *liu* cluster	Pseudomonas Genetic Stock Center
pMO011609	pLA2917 cosmid plus 25 kb of PAO1 genome, including the *atu* cluster	Pseudomonas Genetic Stock Center
pAL-22	pUCP20 with 7.42-kb HindIII-EcoRV DNA fragment containing *liuABCDE* genes from pMO013850 cosmid	5
pAL-23	pUCP20 with 7.84-kb KpnI-XbaI DNA fragment containing *atuCDEF* genes from pMO011609 cosmid	This work
pLP170	*lacZ* transcriptional fusion vector able to replicate in *P. aeruginosa*	23
pANP2	P2 *liuB*::*lacZ* fusion derived from pLP170	5

carbenicillin, 100 μg ml^{-1}; gentamicin (Gm), 100 μg ml^{-1}; and tetracycline (Tc), 100 μg ml^{-1}. Antibiotic concentrations used for *E. coli* strains were as follows: ampicillin, 100 μg ml^{-1}; Gm, 20 μg ml^{-1}; and Tc, 15 μg ml^{-1}.

Genetic tests. Construction of double mutants (Table 1) was done using triparental conjugation among the *P. aeruginosa* PAO1 mutant strain PAO *atuF* (open reading frame [ORF] PA2891) (13), the pRK2013 helper plasmid (8), and one of the pKAAA, pKAAB, and pKAAL plasmids, which contain the disrupted *liuD* (ORF PA2012), *liuB* (ORF PA2014), and *liuE* (ORF PA2011) genes, respectively, as described by Díaz-Pérez et al. (5). Transconjugants were selected on plates with Tc and Gm, and their growth ability on M9 medium with the appropriate carbon source was evaluated. For genetic complementation, the complete wild-type operons were obtained from a cosmid genomic library of *P. aeruginosa* PAO1 (Pseudomonas Genetic Stock Center of the Pseudomonas Genome Project [PGP]). Cosmids pMO013850 (containing the *liu* cluster) and pMO011609 (containing the *atu* cluster) carried a 25-kb PAO1 chromosomal DNA fragment. Fragments of 7.4 and 7.8 kb containing the *liu* and *atu* clusters were subcloned into the pUCP20 vector, rendering the pAL-22 and pAL-23 plasmids, respectively. Genetic complementation was assayed either by conjugation or by heat shock transformation of mutant strains (5), using the wild-type operons from the plasmids mentioned above (Table 1). Recombinants were selected on plates with the appropriate antibiotics and subjected to growth tests.

Quantification of β-galactosidase. The β-galactosidase activities of *P. aeruginosa* strains harboring a LacZ transcriptional fusion were quantified in cultures grown at 30°C to the exponential phase (optical density at 600 nm of 0.6) in M9 medium supplemented with glucose (0.2%, wt/vol); cells were harvested by centrifugation, washed twice with M9 salts solution, and suspended in M9 salts. The inducer compound at 0.005% or 0.2% (wt/vol) was added to the cell suspensions and incubated at 30°C, aliquots were taken at intervals, and β-galactosidase activity was measured as described previously (27).

Detection of biotin-containing proteins. Samples of 100 μg of total protein of cell-free extracts from cultures of *P. aeruginosa* strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were blotted using avidin-horseradish peroxidase (HRP) (Bio-Rad) as indicated by the provider. HRP color development was done using 4-chloro-1-naphtol (Sigma) and H_2O_2. Biotinylated sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein standards (Bio-Rad) were used as molecular markers.

Determination of carboxylase activity. Cultures of the *P. aeruginosa* PAO1 strain were grown in 50 ml of M9 with 0.2% of the appropriate carbon source for 48 h to 30°C with shaking. Cells were harvested by centrifugation and washed with 50 ml 100 mM K_2HPO_4, pH 8.0. Pellets were suspended in 5 ml of the same buffer, disrupted by sonication, and centrifuged for 10 min at 15,000 × *g* and 4°C to eliminate unlysed cells. Protein content was determined as described previously (27). Acetyl-CoA carboxylase (ACCase), propionyl-CoA carboxylase (PCCase), and MCCase activities in the crude extracts were measured by the incorporation of $^{14}CO_2$ into acid nonvolatile material as previously described (10, 25). The reaction mixture contained 100 mM K_2HPO_4 (pH 8.0), 300 μg bovine serum albumin, 10 mM ATP, 10 mM $MgCl_2$, 50 mM $NaH^{14}CO_3$ (specific activity, 1.96 GBq/mmol; 53 mCi/mmol; Amersham), cell extract (300 μg of protein), and 0.5 mM of the appropriate substrate (acetyl-CoA, propionyl-CoA, or 3-methylcrotonyl-CoA, from Sigma Co.) in a total reaction volume of 100 μl. The reaction was started by the addition of $NaH^{14}CO_3$, the mixture was incubated at 37°C for 10 min, and the reaction was stopped by adding 200 μl of 6 M HCl. The contents of the tubes were then evaporated to dryness at 90°C, and the residue was suspended in 100 μl of distilled water. Radioactivity was quantified using a liquid scintillation counter (Beckman LS Analyzer LS6000IC). Nonspecific CO_2 fixation by crude extracts was assayed in the absence of substrate.

Metabolite analyses and degradation rates. Strains were grown in 100 ml of M9 medium with 0.2% (wt/vol) succinic acid and 0.05% (wt/vol) casein peptone at 30°C with shaking for 18 h. Cells were harvested by centrifugation and washed twice with M9 salts solution. The pellet was suspended in the original volume with M9 salts, and citronellol was added to a final concentration of 0.1% (vol/

vol). The suspension was incubated at 30°C with shaking, and aliquots of 25 ml were withdrawn at intervals and centrifuged at 10,000 × *g* for 10 min. Supernatants were saturated with NaCl (7.5 g) and extracted three times with 10 ml of ethyl acetate. Samples were collected, dried with anhydrous Na_2SO_4, concentrated by evaporation in a fume hood at 50°C, and suspended in 500 μl methanol. Samples of 1 μl were analyzed by gas chromatography (GC) (Perkin-Elmer Auto System gas chromatograph), using an Agilent/J&W HP-FFAP GC column (length, 30 m; inner diameter, 0.25 mm; film, 0.25 μm). The analyses were carried out with a 200°C injection temperature and a 250°C detection temperature, with a method start to 60°C for 2 min, an increase to 135°C (25°C/min), and then an increase to 210°C (5°C/min). Commercial citronellol, citral, citronellic acid, and geranic acid mix isomers were used in the GC as standard identification compounds.

Leucine and citronellol degradation rates. Strains were grown in 20 ml of M9 medium with 0.2% (wt/vol) citric acid plus 0.2% (wt/vol) leucine and 0.075% (vol/vol) citronellol at 30°C with shaking for 18 h. Cells were harvested by centrifugation and washed twice with M9 salts solution. The pellet was suspended in the original volume with M9 salts with leucine plus citronellol (0.2% each). The bacterial suspension was incubated at 30°C with shaking, and aliquots of 1 ml were withdrawn at intervals and centrifuged at 10,000 × *g* for 10 min. Leucine and citronellol concentrations in supernatants were determined by high-pressure liquid chromatography. Samples of 50 μl were analyzed using a C_{18} reverse-phase analytical column (Alltech), carried out with isocratic runs using deionized water for 20 min for leucine and with a water-acetonitrile mix as the mobile phase for 5 min (60:40), 10 min (20:80), and 10 min (0:100) for citronellol quantification runs. Detection was done at a UV wavelength of 210 nm, using a Perkin-Elmer high-pressure liquid chromatograph.

Sequence analysis. The nucleotide and amino acid sequences analyzed are designated ORFs PA2011 to PA2016 and ORFs PA2888 to PA2891 in PGP (http://www.pseudomonas.com) (30) and were renamed the *liu* and *atu* clusters, respectively (12).

Phylogenetic analysis. Protein sequence data were obtained from the ortholog clusters of the SSBD KEGG (Kyoto Encyclopedia of Genes and Genomes) and MBGD (Microbial Genome Database for Comparative Analysis) databases by using LiuD (PA2012) and AtuF (PA2891) as queries. Redundant protein sequences were removed, resulting in 201 protein sequences. Progressive multiple protein sequence alignment was calculated with the ClustalW version 1.83 software (11), and the alignment was refined with the MUSCLE version 3.51 program (24) and was later corrected according to the results of gapped BlastP (21). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2.1 (16) using distance-based methods, i.e., unweighted pair group method with arithmetic mean (UPGMA), neighbor joining (NJ), and minimum evolution (ME), with the Poisson correction distance method and with gaps treated by pairwise deletion. Confidence limits of branch points were estimated by 1,000 bootstrap replications.

RESULTS AND DISCUSSION

Identification of the *atu* cluster. In a previous work, we characterized the *P. aeruginosa gnyRDBHAL* gene cluster, which is involved in AMTC catabolism; it was renamed the *liuRABCDE* cluster because of its involvement in leucine and isovalerate utilization (12). From a library of about 1×10^6 *P. aeruginosa* Gm^r mutants generated by transposition (5), about 5,000 mutants were screened for their ability to grow on AMTC as the sole carbon source. Genomic regions interrupted by the transposon in the mutants were cloned in the pBluescript SK vector, and the flanking DNA fragments were sequenced (data not shown). Sequence analysis from strain PAM1529 showed that transposon insertion occurred in the PA2891 ORF from the PGP database. By further analysis of the regions flanking ORF PA2891 in the PAO1 genome, a putative *atu* cluster containing ORFs PA2888, PA2889, PA2890, and PA2891 was located, which corresponded with the *atu* cluster named for its involvement in AMTC utilization (12).

Sequence analysis of the *atu* cluster. Nucleotide sequence analysis of the *atu* cluster showed that it could be constituted by eight probable structural genes, the ORFs PA2886 to PA2893 (Fig. 2). ORFs PA2888 (AtuC) and PA2891 (AtuF) showed similarity with the β and α subunits of the acyl-CoA carboxylases family (ACCase, PCCase, and MCCase) and 63% and 64% of similarity with the LiuB (PA2014) and LiuD (PA2012) proteins from *P. aeruginosa*, respectively. AtuF showed the ATP-binding site (GxGxxG), the sequence implicated in CO_2 fixation (RDCS), the catalytic site of the biotin-dependent carboxylase family (EMNTR), and the biotin-carboxyl carrier domain (AMKM) (5, 14, 15, 29). AtuC showed the acyl-CoA-binding and the carboxybiotin-binding domains (5, 14, 29). These data suggest that the *atuC* and *atuF* genes could encode the subunits of a novel acyl-CoA carboxylase, as also proposed by Hoschle et al. (12).

ORF PA2889 (AtuD) showed high amino acid sequence similarity with acyl-CoA dehydrogenases and 34% of identity with the LiuA protein of *P. aeruginosa*. ORF PA2890 (AtuE) showed similarity with enoyl-CoA hydratase/isomerase and 38% identity with the LiuC protein of the PAO1 strain. ORFs PA2886, PA2887, and PA2892 showed similarity with predicted hypothetical proteins and short-chain dehydrogenase family proteins, respectively, whereas ORF PA2893 (AtuH) showed similarity with very-long-chain acyl-CoA synthetases, suggesting that these three ORFs could be related to the *atu* cluster, although mutagenesis results (see below) do not indicate that this is so. These data suggest that the *atuCDEF* gene may code for products homologous to those of the *liuABCD* genes (Fig. 2).

***atu*/*liu* homologous clusters among *Pseudomonas* species.** The amino acid sequences encoded by the most conserved gene homologs (AtuC, AtuD, AtuE, and AtuF) corresponding to several *Pseudomonas* species were analyzed by multiple alignments. Probable homologs were found in genomes from *P. putida* KT2440, *P. fluorescens* PfO-1, *Pseudomonas syringae* pv. tomato D3000, and *P. syringae* pv. syringae B728a (Fig. 2A). Interestingly, only one homolog cluster was found in these genomes, making evident the catabolic versatility of *P. aeruginosa*, where two homolog clusters were found.

The resultant alignments and phylogenetic tree showed that the *liu* cluster products from *P. aeruginosa* had more similarity with the products of the *liu* homolog clusters from the other *Pseudomonas* species (77.2 to 79.5%) than with the products of the *atu* cluster from *P. aeruginosa* (41.5%) (Fig. 2B). This tendency was also observed when the ORFs were analyzed separately, where amino acid identities ranged from 65% to 94% with the *liu* cluster versus 34% to 50% with the *atu* cluster (Fig. 2A). These analyses showed that the homologous proteins encoded by the *liu* genes are conserved in both amino acid sequence and genetic arrangement in the *Pseudomonas* species studied.

Interestingly, a *liuE* homolog gene was not found in the *atu* cluster or elsewhere in the PAO1 genome (Fig. 2A), suggesting that LiuE may have a dual function, being used in both the AMTC and the leucine catabolic pathways. This is the first study to the genetic level in which a hydroxymethyl-glutaryl-CoA lyase (HMG-CoA lyase) homolog (LiuE) has been found to be involved in both AMTC and leucine catabolism in bacteria (see below).

Growth analysis of *atu* and *liu* mutants. The *P. aeruginosa* PAO1 wild-type strain was able to grow on leucine or isoleucine as well as on citronellol as the sole carbon and energy source, while *P. fluorescens* and *P. putida* strains were unable to

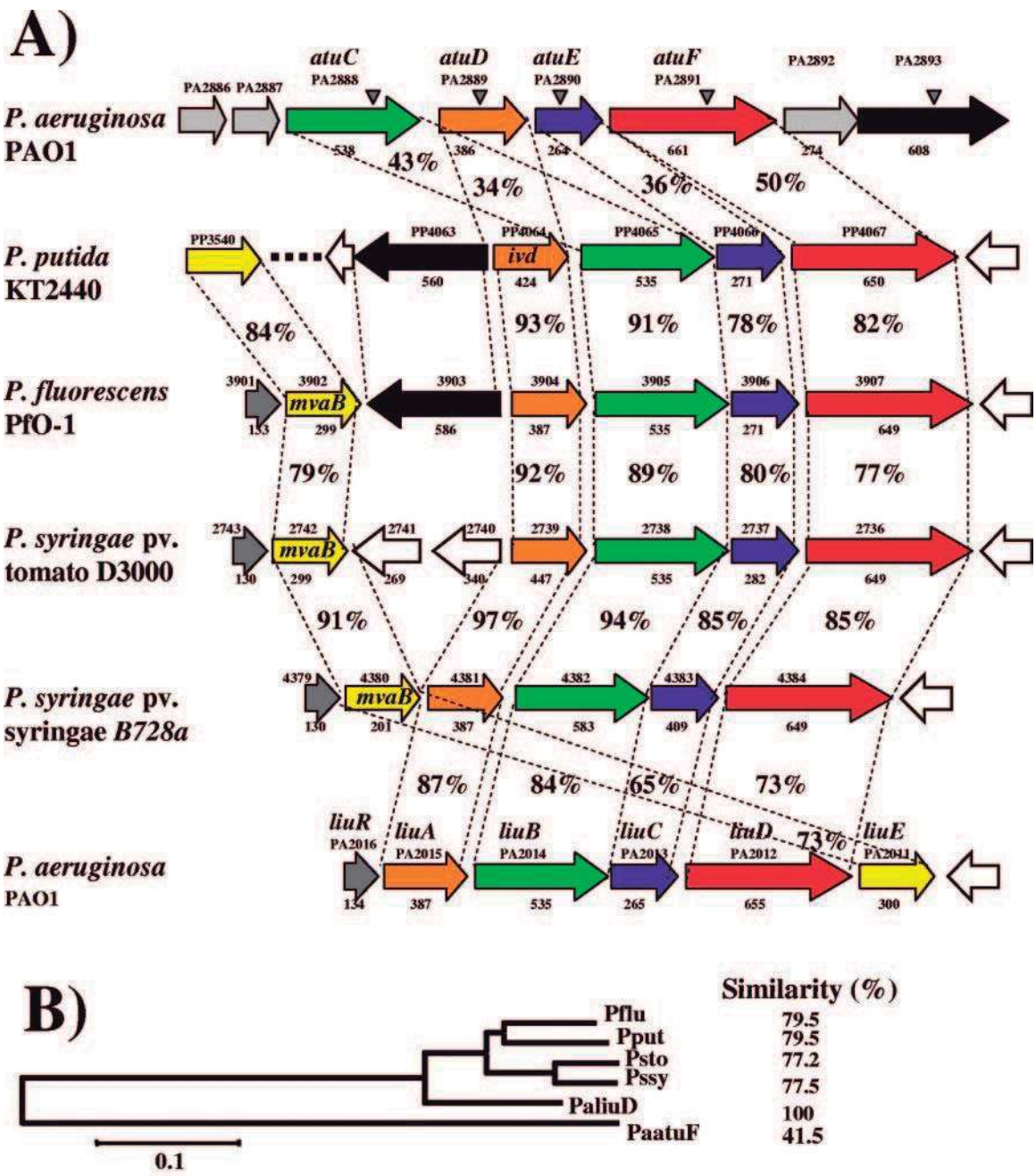

FIG. 2. Sequence analysis of products encoded in the *atu* and *liu* gene clusters. (A) Genetic arrangement of the *atu*/*liu* homolog clusters from *Pseudomonas* species. The species and strain names are shown at the left. ORF arrangement and transcription direction are shown by arrows. Locations of transposon insertions in the *P. aeruginosa* mutants are indicated with shaded arrowheads. Numbers above arrows show the ORF number assigned in the respective genome sequencing project. Identified genes are also shown. The amino acid number of the ORF is indicated below the arrows. Corresponding ortholog proteins are indicated by the same color. Orange, LiuA; green, LiuB; blue, LiuC; red, LiuD; yellow, lyase (LiuE); black, PA2893.The percentages of amino acid identity between upper and lower ORFs are indicated. (B) Phylogenetic tree of the Atu/Liu homolog proteins of *Pseudomonas* species. The amino acid sequences of LiuABCD and AtuDCEF were put together as one sequence and aligned with the homologous sequences from the strains indicated. The alignment was done using the Clustal W software, and the tree was done by the neighbor joining in the Mega2 software with a bootstrap of 1,000 replicates. The numbers indicate the percent similarities between the amino acids sequences aligned. Pflu, *P. fluorescens* PfO-1; Pput, *P. putida* KT2440; Psto, *P. syringae* pv. tomato D3000; Pssy, *P. syringae* pv. syringae B728a; PaatuF, *P. aeruginosa* PAO1 *atu* cluster; PaliuD, *P. aeruginosa* PAO1 *liu* cluster. The evolutionary distance scale bar is shown at the bottom.

TABLE 2. Growth phenotypes of *Pseudomonas* strains

Strain	Growth in[a]:			Doubling time (h)[e]
	Citronellol	L-Leucine	L-Isoleucine	
P. aeruginosa PAO1SM	+++	+++	+++	8.2
P. fluorescens L1	−	+++	+++	ND[f]
P. putida	−	+++	+++	ND
PAO2893[b]	+++	+++	+++	8.0
PAO *atuD*[b]	−	+++	+++	7.5
PAO *atuE*[b]	−	+++	+++	11.2
PAO *atuC*[b]	−	++	+++	13.1
PAO *atuF*[b]	−	+	+++	13.5
PAO *atuF*(pAL-22)[b]	−	++	+++	6.5
PAO *atuF*(pAL-23)[b]	+++	+++	+++	7.0
PAM1529 (*atuF* mutant)[c]	−	+++	+++	13.1
PAM1529(pAL-22)[c]	−	+++	+++	6.5
PAM1529(pAL-23)[c]	+++	+++	+++	6.5
PAE80 (*liuD* mutant)[c]	−	−	+++	ND
PAE80(pAL-22)[c]	+++	+++	+++	9.0
PAE80(pAL-23)[c]	−	−	+++	ND
PAM *liuD*[d]	−	−	+++	ND
PAM *liuD*(pAL-22)[d]	+++	+++	+++	9.7
PAM *liuD*(pAL-23)[d]	−	−	+++	ND
PAO *liuD*[b]	−	+++	+++	ND
PAM *liuA*[d]	−	−	+++	ND
PAM *liuB*[d]	−	−	+++	ND
PAM *liuE*[d]	−	−	+++	ND
PAO *atuF liuD*	−	−	+++	ND
PAO *atuF liuB*	−	−	+++	ND
PAO *atuF liuE*	−	−	+	ND
PAO *atuC liuE*	−	−	+	ND

[a] The strains were inoculated on M9 plates with the compounds indicated as sole carbon sources and incubated for 48 h at 30°C as described in Materials and Methods. +++, good growth; ++, medium growth; +, low growth; −, no growth.

[b] Mutant obtained by Jacobs *et al.* (13) (no polar effects over downstream gene expression).

[c] Mutant described in this work (polar effects over downstream gene expression).

[d] Mutant obtained by homologous recombination (polar effects over downstream gene expression).

[e] Doubling times of cultures grown in M9 minimal liquid medium with leucine (0.2%, wt/vol) as the sole carbon source, incubated at 30°C with shaking at 150 rpm. The growth was measured by optical density at 600 nm and calculated in the exponential growth phase.

[f] ND, not determined.

grow on citronellol (Table 2). The *atu* mutants with an outward-facing promoter, which reduces polar effects on downstream gene expression (PAO *atuC*, PAO *atuD*, PAO *atuE*, and PAO *atuF*), were unable to grow on citronellol but were able to grow on leucine, except for the PAO *atuC* and PAO *atuF* mutants, whose growth was impaired. Growth on leucine, isoleucine, and citronellol was not affected in the PAO2893 mutant, which contains a transposon insertion in ORF PA2893 (Table 2), suggesting that this ORF is not involved in leucine or AMTC catabolic pathways.

Mutants obtained with the mutagenesis system that provokes polar effects over downstream gene expression by transposition or homologous recombination, i.e., PAE80 (affected in the *liuD* gene), PAM *liuA*, PAM *liuB*, PAM *liuD*, and PAM *liuE*, were unable to grow on both citronellol and leucine, whereas PAM1529 (affected in the *atuF* gene) was unable to grow on citronellol. When the PAM1529, PAE80, and PAM *liuD* mutants were transformed with plasmid pAL-22 (contains the *liu* cluster) or pAL-23 (contains the *atu* cluster), only pAL-23 complemented the growth on citronellol in the PAM1529 mutant, while in the PAE80 and PAM *liuD* mutants, only pAL-22 restored the ability to grow on both leucine and citronellol (Table 2). In contrast, for the PAO *atuF* mutant, unable to grow on citronellol and impaired in leucine growth, leucine growth was improved with both plasmids, but only with pAL-23 was growth on citronellol recovered (Table 2). These results suggest that both the *atu* and *liu* clusters are related to the ability to grow on both citronellol and leucine and also suggest that their respective acyl-CoA carboxylases encoded in the *atu* and *liu* clusters may be shared in both AMTC and leucine catabolism. In addition, all single mutant strains tested were able to grow on isoleucine (Table 2), suggesting that the phenotypes shown in the mutants were exclusively associated with leucine or citronellol metabolism.

The phenotypes of double mutants affected in the *atu* and *liu* cluster genes (*atuF liuD*, *atuF liuB*, *atuF liuE*, and *atuC liuE* mutants) showed a clear deficiency in growth on both AMTC and leucine (Table 2). The impaired growth on isoleucine in the last two strains is still unclear. These results show that the *atu* and *liu* clusters are absolutely necessary for AMTC catabolism and that mutations in some of their genes affect leucine utilization.

Growth rates of the *atu* and *liu* mutants. The PAO1 strain grown on leucine as the sole carbon and energy source had a doubling time of 8.2 h, whereas the PAO *atuE*, PAO *atuC*, and PAO *atuF* mutants showed impaired growth in leucine, with doubling times of 11.2, 13.1, and 13.5 h, respectively (Table 2). When the PAO *atuF* mutant strain was transformed with the pAL-22 (containing the *liu* cluster) or pAL-23 (containing the *atu* cluster) plasmid, the growth rate in leucine was even better in the transformed mutants than in the wild-type strain, showing doubling times of 6.5 h and 7.0 h, respectively (Table 2), probably due to protein overexpression. For the PAE80 strain (*liuD* gene mutant), leucine growth was recovered with the pAL-22 plasmid, in the same proportion as with the PAO1 strain, but not with pAL-23 (Table 2). As expected, the *atuF liuD*, *atuF liuB*, *atuF liuE*, and *atuC liuE* double mutants and the PAM *liuE* mutant did not grow on leucine (Table 2). The leucine complementation results for both the *atuF* and *liuD* mutants with the *atu* or *liu* clusters (contained in the pAL-23 or pAL-22 plasmid, respectively) suggest that the leucine catabolic pathway could share gene products. The PAM1529 (*atuF* mutant) and PAE80 (*liuD* mutant) strains were unable to grow on citronellol, but they recovered the growth on citronellol in similar proportion as the PAO1 strain when were transformed with the pAL-23 and pAL-22 plasmids, respectively (Table 2).

In addition, the PAM *liuE* mutant was complemented in its growth on both leucine and citronellol by the pMO013850 cosmid (containing the *liu* cluster), but not by the pMO011609 cosmid (containing the *atu* cluster) (data not shown), confirming that LiuE (an HMG-CoA lyase homolog) is located in the *liu* cluster in *P. aeruginosa* and that it is absolutely necessary for the degradation of AMTC and leucine.

When cosmids pMO011609 and pMO013850 were transferred to *P. putida* and *P. fluorescens* strains (both wild-type strains are able to grow on leucine [Table 2]), neither strain was able to grow on citronellol (data not shown), suggesting that additional genes, not contained in their genomes or in the *liu* and *atu* clusters, are required for AMTC degradation.

Metabolite accumulation. *P. aeruginosa liuD* and *liuB* mutants have been shown to accumulate citronellic and geranic acids when incubated with citronellol (5). The *liuD* mutant grown in the presence of citronellol accumulated principally citronellic acid, while the *atuF* mutant accumulated both citronellic and geranic acids, compared to the wild-type strain (Fig. 3). Accumulation of citronellic and geranic acids in *liuD* and *atuF* mutants suggests that the activities of both GCCase and MCCase are indispensable for AMTC degradation (Fig. 1).

Leucine and citronellol assimilation rates. Assimilation kinetics of the *P. aeruginosa* wild-type PAO1 strain showed utilization of leucine and citronellol in liquid cultures with these compounds as carbon sources of 48% and 62%, respectively, after 24 h of incubation (data not shown). Utilization of leucine and citronellol by the PAO *liuD* mutant was 3% and 40%, and that by the PAO *atuF* mutant was 17% and 4%, respectively (data not shown). The assimilation of leucine by the PAO *liuD* mutant was slower than that of the PAO *atuF* mutant. In contrast, the ability to assimilate citronellol was more severely affected in the PAO *atuF* mutant than in the PAO *liuD* mutant. These results further confirm that the *liu* cluster is principally involved in leucine assimilation and the *atu* cluster in citronellol catabolism and also suggest that both clusters are involved in leucine and AMTC catabolic pathways and that the *liu* and *atu* clusters probably share gene products. These results are in disagreement with those described by Hoschle et al. (12), who concluded that the GCCase is needed only for utilization of AMTC and that the MCCase cannot replace GCCase (12).

Expression of *liuD* and *atuF* genes. Western blotting analysis from cultures of PAO1 grown in minimal medium with citronellol as the sole carbon source usually shows four major biotinylated proteins with molecular masses of 73, 70, 62, and 22 kDa (5) (Fig. 4a). The 73- and 70-kDa proteins have been proposed to correspond to α-GCCase and α-MCCase subunits, respectively (5). However, Hoschle et al. (12) recently reported that *P. aeruginosa* PAO1 showed three biotinylated proteins (74, 71, and 63 kDa) when grown on citronellate, suggesting by trypsin fingerprint analysis that these corresponded to the ORFs PA2012 (71.28 kDa), PA2891 (71.74 kDa), and PA5435 (66.09 kDa), respectively. The 74- and 71-kDa proteins were named LiuD and AtuF, respectively (12). When the wild-type PAO1 strain was grown on glucose, the LiuD (73 kDa) and AtuF (70 kDa) proteins were not observed (Fig. 4A, lane 2); with isoleucine as the sole carbon and energy source, a slight expression of LiuD was observed (Fig. 4A, lane 3). With leucine, LiuD, but not AtuF, was present (Fig. 4A, lane 4), suggesting that LiuD is related to leucine and isoleucine metabolism. When citronellol was used for growth, both the LiuD and AtuF proteins were overexpressed (Fig. 4A, lane 5). These results show that citronellol, leucine, and isoleucine, in decreasing order, were able to induce the expression of the LiuD protein, whereas only citronellol was able to induce the expression of both the LiuD and AtuF proteins, confirming further that both *liu* and *atu* cluster genes are required for AMTC catabolism. In addition, in extracts obtained from cultures of the PAO1 strain grown on glucose plus either leucine or citronellol, the expression levels of the LiuD and AtuF proteins were somewhat decreased (Fig. 4A, lanes 6 and 7), sug-

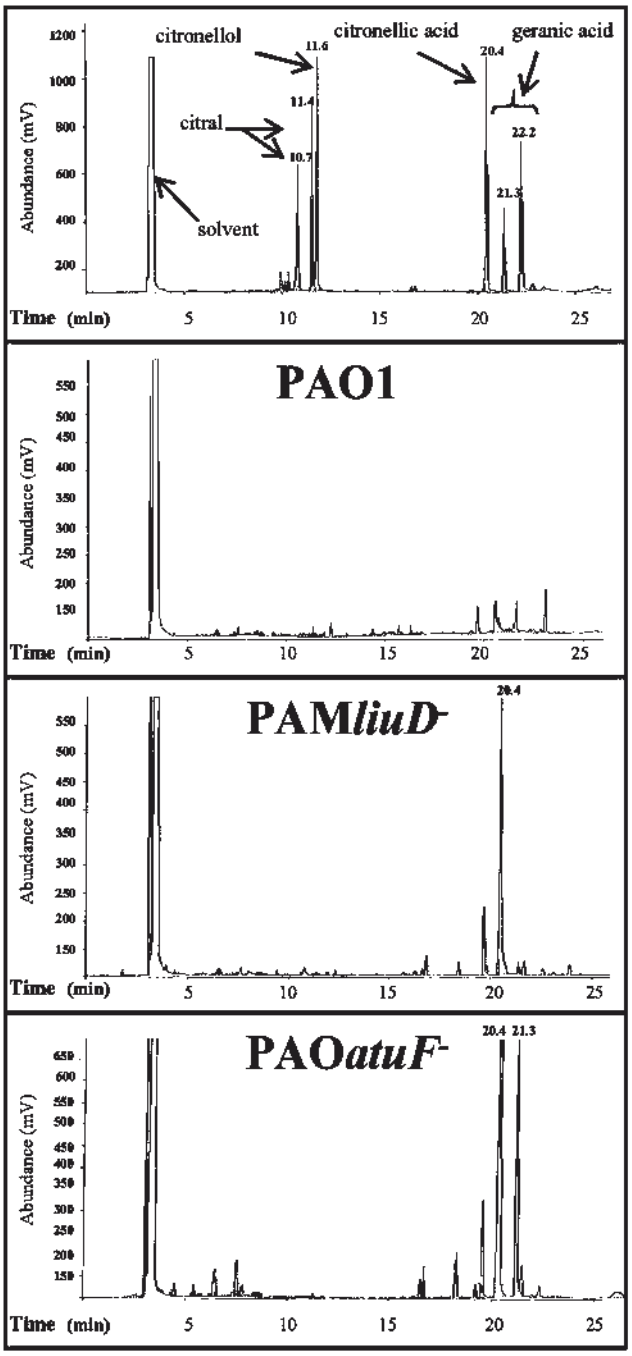

FIG. 3. Metabolite accumulation in cultures of *P. aeruginosa* grown in citronellol. Cultures were grown in M9 medium with succinic acid as the carbon source for 18 h, and citronellol was then added. The metabolites were extracted from the supernatants and analyzed at 48 h after the addition of citronellol by gas chromatography as described in Materials and Methods. Commercial citral (10.7 and 11.4 min), citronellol (11.6 min), citronellic acid (20.4 min), and geranic acid mix isomers (21.3 and 22.2 min) were used as standard compounds. Peaks with retention times of 20.4 and 21.3 min correspond to citronellic acid and geranic acid, respectively.

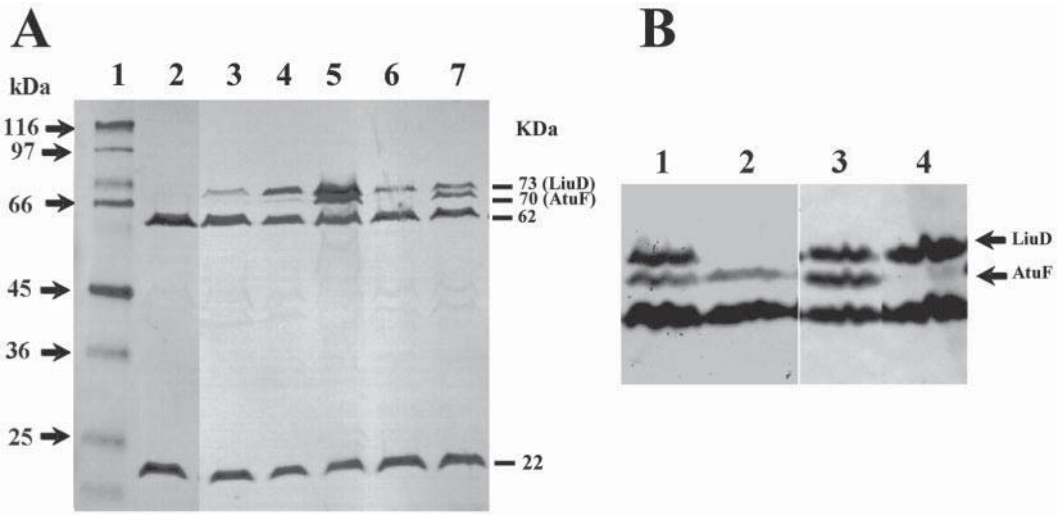

FIG. 4. Western blot analysis of biotinylated proteins from *P. aeruginosa* PAO1 derivatives. (A) Lanes: 1, molecular masses of protein standards; 2 to 7, cell extracts from the PAO1 strain grown on glucose (lane 2), isoleucine (lane 3), leucine (lane 4), citronellol (lane 5), glucose plus leucine (lane 6), and glucose plus citronellol (lane 7). Proteins of 73, 70, 62, and 22 kDa identified with avidin-HRP conjugate, corresponding to biotinylated subunits from alpha subunits of geranyl-CoA carboxylase (AtuF), 3-methylcrotonyl-CoA carboxylase (LiuD), a putative acyl-CoA carboxylase, and acetyl-CoA carboxylase, respectively, are indicated. (B) Biotinylated proteins from cultures grown in glucose plus citronellol. Lanes: 1 and 3, PAO1 wild-type strain; 2, PAM *liuD* mutant; 4, PAO *atuF* mutant. The positions of the LiuD and AtuF proteins are indicated.

gesting that glucose exerts a moderate catabolite repression effect on the expression of both *liuD* and *atuF* genes.

In extracts from the PAM *liuD* and PAO *atuF* mutants grown on citronellol plus glucose, only the 70- and 73-kDa proteins were detected, respectively (Fig. 4B). This confirms that the LiuD and AtuF proteins are encoded by the *liuD* and *atuF* genes, respectively, as was also found by trypsin fingerprint protein analysis (12). These results suggest that the LiuD protein corresponds to the alpha subunit of MCCase (α-MCCase) and that the AtuF protein corresponds to α-GCCase.

The expression of the *liu* cluster in PAO1 was also tested using a *liuB*::*lacZ* transcriptional fusion. The reporter gene was induced threefold by either leucine or 3-methylcrotonoic acid (a compound related to the leucine catabolic pathway), whereas citronellol induced it about sixfold, compared to the glucose-grown cultures (Fig. 5). This induction was also reflected at the translational level, since the LiuD protein showed a higher level in cultures grown on citronellol than in those grown on leucine (Fig. 4A). These results show that citronellol is a better inducer of the *liu* cluster than leucine.

Carboxylase activity. In cell extracts from *P. aeruginosa* PAO1 obtained from cultures grown on glucose or on glucose plus citronellol or leucine, the MCCase enzymatic activity was low (Table 3). These results are in agreement with our previous finding that glucose exerts catabolite repression on *liuD* expression. The activity of MCCase increased 6-fold in cultures of the PAO1 strain grown on leucine compared to extracts from cultures grown on glucose; however, cultures grown on citronellol showed a 16-fold increase in MCCase activity (Table 3). Growth on citronellol had no significant differences from growth on glucose when the ACCase and PCCase enzyme activities were measured, while growth on glucose plus leucine showed only slight differences (Table 3). These results support our data that the MCCase, but not the ACCase and PCCase activities, are induced by citronellol (best inducer) as well as leucine. The differences in enzymatic activity of MCCase between the extracts from cultures grown on citronellol and those grown on leucine could be due to an increase of the expression level of the MCCase (as it was shown in the Western blot analysis) or to unspecific activity of GCCase by geranyl-CoA or 3-methylcrotonyl-CoA substrates. This has been previously described for *P. citronellolis* (7, 9) and could suggest that the GCCase enzyme (encoded by the *atuCF* genes) may also have MCCase activity.

Phylogenetic analysis. The *liu* cluster products from *P. aeruginosa* PAO1 showed more similarity to the products of the *liu*

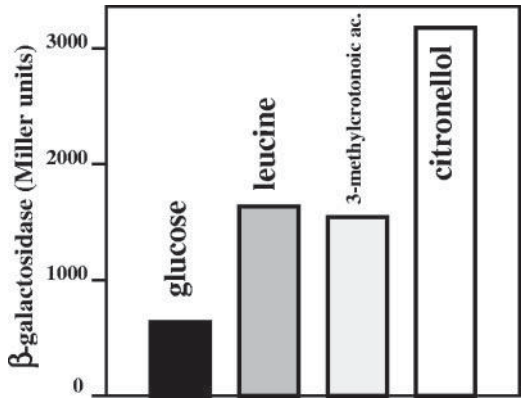

FIG. 5. Induction of the *liuB*::*lacZ* transcriptional fusion. Cultures of the PAO1 strain with plasmid pANP2 (P2*liuB*::*lacZ*) were grown in M9 medium with glucose. β-Galactosidase activity was measured after the addition of the inducer compounds glucose (0.2%), leucine (0.005%), 3-methylcrotonoic acid (0.005%), and citronellol (0.005%). Data given correspond to Miller units after 3 h of induction with the compound indicated and are the averages of two independent experiments done in duplicate; the standard deviation was less than 5% of the given value.

TABLE 3. Carboxylase activity in *P. aeruginosa* PAO1 cell extracts

Carbon source[b]	Carboxylase activity[a] (U/mg of protein) on substrate:		
	3-Methylcrotonyl-CoA	Acetyl-CoA	Propionyl-CoA
Glucose	82.0	36.2	47.8
Glucose + leucine	97.0	143.0	82.0
Glucose + citronellol	45.1	50.0	44.0
Leucine	492.0	ND[c]	ND
Citronellol	1,312.0	73.8	66.9

[a] Enzymatic quantification was carried out as described in Materials and Methods. One unit of enzyme activity catalyzed the incorporation of 1 μmol of ^{14}C into acid-stable product/min. Data given are the averages of two quantifications in duplicate; the standard deviation was less than 10%.

[b] Cell extracts were grown on minimal medium M9 with the carbon source indicated.

[c] ND, not determined.

homologs from other *Pseudomonas* species (also called *mcc* homologs) than to the proteins encoded in the *atu* homologous genes from PAO1 (Fig. 2). Amino acid sequence alignments and phylogenetic analysis of the more important enzymes of the pathway, LiuD/AtuF, were done (Fig. 6). The phylogenetic tree of the LiuD/AtuF (α-MCCase/α-GCCase) proteins with their orthologs showed five phylogenetic groups of the biotinylated subunit of acyl-CoA carboxylases: ACCase, PCCase, MCCase, pyruvate carboxylases, and urea carboxylases (see the supplemental material). The LiuD and AtuF proteins from *P. aeruginosa* were located in the MCCase root, as shown in Fig. 6A. The LiuD protein from *P. aeruginosa* PAO1 was phylogenetically related to the MccA proteins of *P. putida* KT2440, *P. fluorescens* PfO-1, *P. syringae* pv. tomato D3000, and *P. syringae* pv. syringae B728a (Fig. 6A). Further analysis of the MCCase root showed that the similarities of AtuF and LiuD to their homologs from *Rhizobiaceae* of the *Alphaproteobacteria*, such as *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*, were higher than those to the LiuD protein from *P. aeruginosa* itself, which is classified in the *Gammaproteobacteria* root (Fig. 6B). In these bacteria the *liu* homolog cluster could be involved in leucine catabolism, while both the *liu* and *atu* clusters in *P. aeruginosa* are involved in the metabolism of both leucine and AMTC, as described above.

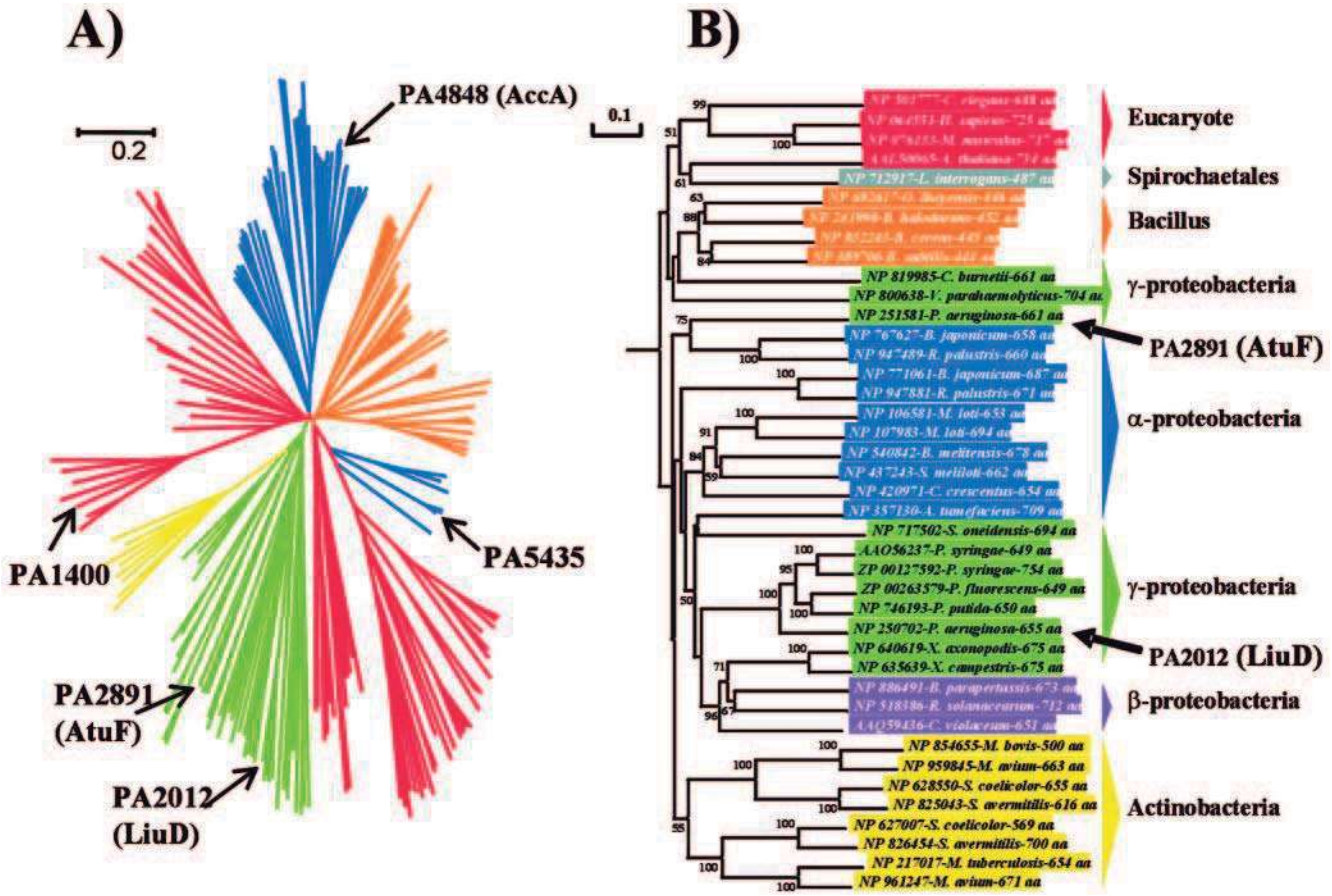

FIG. 6. Phylogenetic trees of AtuF/LiuD protein orthologs. Trees were obtained by the UPGMA, NJ, and ME methods in the Mega2 package as described in Materials and Methods, and the NJ tree is shown. (A) The tree shows the five phylogenetic groups of the biotinylated subunits of acyl-CoA carboxylases aligned; the probable functions of the ortholog proteins are indicated in the phylogenetic groups, in agreement with some characterized carboxylases: acetyl-CoA carboxylases (blue), propionyl-CoA carboxylases (orange), 3-methylcrotonyl-CoA carboxylases (green), pyruvate carboxylases (red), and urea carboxylases (yellow). Acyl-CoA carboxylases from *P. aeruginosa* PAO1 are shown by arrows. (B) Phylogenetic tree of MCCase root constructed with the 41 proteins showing the higher phylogenetic relationship. The AtuF and LiuD proteins of *P. aeruginosa* are shown by arrows. Bootstrap values of higher than 50% obtained by the UPGMA, NJ, and ME methods are shown. Blue, *Alphaproteobacteria*; purple, *Betaproteobacteria*; green, *Gammaproteobacteria*; yellow, *Actinobacteria*; orange, *Bacillus*; gray, *Spirochaeta*; red, eukaryote.

These results further suggest that the current *atu* cluster from *P. aeruginosa* PAO1 probably originated by horizontal transfer from bacteria of the *Rhizobiaceae* (from the *Alphaproteobacteria* root) that contain a GC percentage (64.66 and 65.53% for *B. japonicum* and *R. palustris*, respectively) close to that of *P. aeruginosa* (67.14%). Also, codon usage and codon frequency among these bacteria are highly similar (codon usage database, http://www.kazusa.or.jp/codon/). Genetic transfer has been shown to occur in several examples of the evolution of catabolic pathways in bacteria (31, 34).

In conclusion, our results suggest that both the *atu* and *liu* clusters encode the enzymes involved in the AMTC and leucine catabolic pathways, and probably both carboxylase enzymes (encoded by the *atuCF* and *liuBD* genes) have principally GCCase and MCCase activities, respectively. In addition, the *atu* cluster from *P. aeruginosa* PAO1 lacks an HMG-CoA lyase homolog (LiuE), suggesting a dual function for this enzyme, catalyzing the deacetylation of both 3-hydroxy-γ-carboxygeranyl-CoA in AMTC catabolism and 3-hydroxy-3-methylglutaryl-CoA for leucine breakdown (Fig. 1). Phylogenetic analysis suggests that the *atu* cluster was acquired by a horizontal transfer event, probably from *Rhizobiaceae*, which by a xenologous gene displacement event rendered in *P. aeruginosa* the heterofunctional *liu* and *atu* clusters, implicated in both the AMTC and leucine catabolic pathways.

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Substrate Specificity of the 3-Methylcrotonyl Coenzyme A (CoA) and Geranyl-CoA Carboxylases from *Pseudomonas aeruginosa*▽†

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Biotin-containing 3-methylcrotonyl coenzyme A (MC-CoA) carboxylase (MCCase) and geranyl-CoA (G-CoA) carboxylase (GCCase) from *Pseudomonas aeruginosa* were expressed as His-tagged recombinant proteins in *Escherichia coli*. Both native and recombinant MCCase and GCCase showed pH and temperature optima of 8.5 and 37°C. The apparent $K_{0.5}$ (affinity constant for non-Michaelis-Menten kinetics behavior) values of MCCase for MC-CoA, ATP, and bicarbonate were 9.8 μM, 13 μM, and 0.8 μM, respectively. MCCase activity showed sigmoidal kinetics for all the substrates and did not carboxylate G-CoA. In contrast, GCCase catalyzed the carboxylation of both G-CoA and MC-CoA. GCCase also showed sigmoidal kinetic behavior for G-CoA and bicarbonate but showed Michaelis-Menten kinetics for MC-CoA and the cosubstrate ATP. The apparent $K_{0.5}$ values of GCCase were 8.8 μM and 1.2 μM for G-CoA and bicarbonate, respectively, and the apparent K_m values of GCCase were 10 μM for ATP and 14 μM for MC-CoA. The catalytic efficiencies of GCCase for G-CoA and MC-CoA were 56 and 22, respectively, indicating that G-CoA is preferred over MC-CoA as a substrate. The enzymatic properties of GCCase suggest that it may substitute for MCCase in leucine catabolism and that both the MCCase and GCCase enzymes play important roles in the leucine and acyclic terpene catabolic pathways.

In the corresponding bacterial catabolic pathways, terpenes are converted to *cis*-geranyl coenzyme A (G-CoA) and leucine-isovalerate is converted to isovaleryl-CoA. After four analogous reactions that are common to both pathways, the final products of terpene degradation are acetyl-CoA and 3-oxo-7-methyl-6-octenoyl-CoA, and those of leucine catabolism are acetyl-CoA and acetoacetate (Fig. 1). After two β-oxidation cycles, 3-oxo-7-methyl-6-octenoyl-CoA yields 3-methylcrotonyl-CoA (MC-CoA), an intermediary of the leucine-isovalerate pathway (Fig. 1). Therefore, the acyclic terpene utilization and leucine-isovalerate pathways converge in the MC-CoA intermediate (1, 10). Two homologous gene clusters that encode the enzymes of the acyclic terpene (*atuABCDEFGH*, for *a*cyclic *t*erpenes *u*tilization) and leucine-isovalerate (*liuRABCDE*, for *l*eucine-*i*sovalerate *u*tilization) catabolic routes have been recently identified in *Pseudomonas aeruginosa* (1, 6, 10, 16) and *Pseudomonas citronellolis* (11). Phylogenetic analysis of the *P. aeruginosa* AtuF α subunit of G-CoA carboxylase (GCCase) suggested that it originated by a horizontal transfer event from alphaproteobacteria to *P. aeruginosa* and may implicate different functions (1).

Key enzymes in both pathways are GCCase, encoded by the *atuC*/*atuF* genes, and MC-CoA carboxylase (MCCase), encoded by the *liuB*/*liuD* genes (Fig. 1). These enzymes are composed of two subunits, i.e., LiuB and AtuC (β subunits) and LiuD and AtuF (α subunits), of their respective MCCase and GCCase enzymes (1, 10, 16). MCCase and GCCase enzymes show two main domains, the acyl-CoA-binding and the carboxybiotin-binding domains, which are implicated in the transfer of a carboxyl group to the acyl-CoA substrate (17). On the other hand, both LiuD and AtuF (α subunits) show four highly conserved domains in the acyl-CoA carboxylases: (i) the ATP-binding site (GGGGKGM), (ii) a CO_2 fixation domain (RDCS), (iii) the catalytic site of the biotin-dependent carboxylase family (EMNTR), and (iv) a biotin-carboxyl carrier domain (AMKM).

It has been suggested that the *P. citronellolis* GCCase carboxylates both G-CoA and MC-CoA, while MCCase carboxylates only MC-CoA (9, 13, 15). Although the genes that encode the enzymes of the acyclic terpene and leucine/isovalerate catabolic pathways in *P. aeruginosa* and *P. citronellolis* have been recently elucidated (1, 10), there is still uncertainty regarding the bifunctionality of the carboxylases involved. Using an indirect method for GCCase and MCCase activity determination (a coupled reaction with pyruvate kinase and lactate dehydrogenase), Förster-Fromme et al. (10) suggested that both GCCase and MCCase showed specific activities for their substrates G-CoA and MC-CoA, respectively. However, data from our group suggest that the two catabolic pathways may share gene products or that some enzymes could show a bifunctional activity (1).

This work was focused on the biochemical characterization of MCCase and GCCase and on elucidation of whether these enzymes can use both MC-CoA and G-CoA as substrates.

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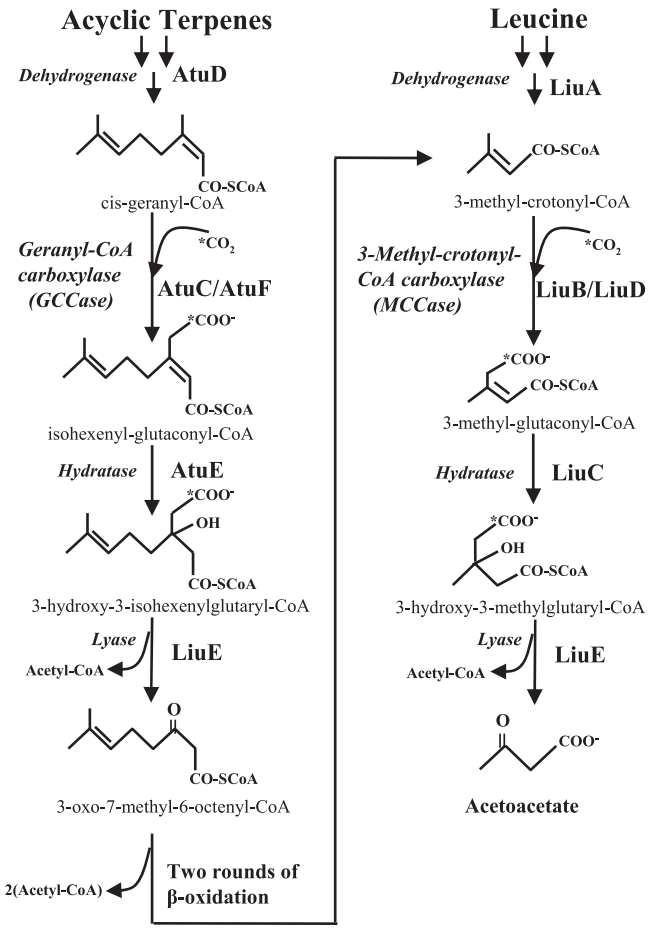

FIG. 1. Participation of GCCase and MCCase in the acyclic monoterpene and leucine catabolic pathways of *P. aeruginosa* PAO1 (1, 10). AtuD, citronellyl-CoA dehydrogenase; AtuC/AtuF, GCCase; AtuE, isohexenylglutaconyl-CoA hydratase; LiuA, isovaleryl-CoA dehydrogenase; LiuB/LiuD, MCCase; LiuC, 3-methylglutaconyl-CoA hydratase; LiuE, 3-hydroxy-3-methyl-glutaryl-CoA (also proposed as 3-hydroxy-3-isohexenylglutaryl-CoA lyase).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this work are *Escherichia coli* TOP10 (Invitrogen), *E. coli* BL21 (22), *E. coli* JM101 (22), and *P. aeruginosa* PAO1SM (25). Plasmids used were pGEM-T Easy (Promega), pTrc His2A and -C (Invitrogen), and pCDFDuet-1 (Novagen). The strains were grown at 30°C in Luria-Bertani (LB) medium or in M9 minimal medium (22). Solid media were prepared by adding 1.5% agar. Strains were grown on M9 minimal medium supplemented with 0.075% citronellol (Merck) as the sole carbon and energy source. The growth of strains on branched-chain amino acids was tested as described previously (20), using 0.3% (wt/vol) L-leucine supplemented with L-valine and L-isoleucine at 0.005% each (obtained from Sigma and Merck Co.). Antibiotics used were streptomycin at 200 μg/ml and ampicillin at 100 μg/ml.

DNA manipulation and cloning of the *atu* and *liu* genes. Genomic and plasmid DNA extraction, restriction enzyme digestion, and agarose gel electrophoresis were carried out by standard methods (22). *P. aeruginosa* genomic DNA was used as the template for PCR amplification. For *liuD* gene amplification the oligonucleotide LiuD1 was used to introduce a 5′ BamHI restriction site upstream of the start codon, and LiuD2 was used to introduce a HindIII restriction site at the 3′ end of *liuD*. For *liuB*, *atuC*, and *atuF* the amplification strategy was the same, using the oligonucleotides LiuB1 and LiuB2, AtuC1 and AtuC2, and AtuF1 and AtuF2. The *atuF* gene was also amplified using the oligonucleotides AtuF3, introducing a BglII site, and AtuF4, introducing a KpnI site (see Table S1 in the supplemental material).

PCR amplification was carried out using Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturer's recommendations. Amplified DNA fragments were cloned into the pGem-T Easy vector, giving plasmids pG*liuB*, pG*liuD*, pG*atuF*, and pG*atuC* with the cloned *liuB*, *liuD*, *atuF*, and *atuC* genes, respectively. Recombinant plasmids were subjected to double digestion at the respective enzyme sites designed in the oligonucleotides described above, and the DNA fragments for the *liuB*, *liuD*, and *atuC* genes were subcloned into the pTrcHis2A plasmid, while the *atuF* gene was subcloned into the pTrcHis2C plasmid, giving the pTrc-*liuB*, pTrc-*liuD*, pTrc-*atuC*, and pTrc-*atuF* plasmids. Additionally, the *atuC* and *atuF* genes were amplified with the AtuC1-AtuC2 and AtuF3-AtuF4 oligonucleotides, respectively; the resulting fragments were cloned into pGem-T Easy and subcloned into the pCDFDuet-1 coexpression vector, giving the pCFC3-*atuFC* plasmid. The recombinant plasmids were transferred to electrocompetent *E. coli* JM101 cells and analyzed by digestion with restriction endonucleases and by DNA sequencing.

Expression, purification, and enzyme reconstitution of recombinant proteins. The plasmids containing the *atu*/*liu* genes were transferred to *E. coli* strain TOP10 or BL21, the cells were cultured on LB medium (400 ml), expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) (0.1 mM), and the cells were incubated for an additional 4 h at 37°C with shaking. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. The bacterial pellet was suspended in 10 ml of buffer (50 mM Tris-HCl, pH 7.4) and disrupted by sonication at 4°C. The proteins were purified from crude extracts according to the His-bind purification kit protocol (Novagen). The resin-protein mixture was washed twice with 1× wash buffer, and the protein was eluted using 1 ml of elution buffer containing 100 mM imidazole.

Reconstitution of MCCase and GCCase was carried out using the subunits purified as described above and denaturing with 6 M urea. The α and β subunits (2 mg each) were mixed and renatured by dialysis using a Spectrum 10-kDa-molecular-mass-cutoff (Fisher Scientific) membrane in a buffer containing 20 mM K_2HPO_4, 20% glycerol, and 0.75 mM dithiothreitol (pH 8.0) for 1 h at 4°C, changing the buffer (100 ml) six times.

G-CoA synthesis. G-CoA was synthesized by the mixed anhydride method of Hajra and Bishop (14). Geranic acid (Sigma-Aldrich) (770 μmol) was dried twice with 500 μl of benzene under a gentle N_2 stream; the same molar amount of butylated hydroxytoluene dissolved in benzene was added, and 400 μl of oxalyl chloride and 800 μl of benzene were added and flushed with N_2. The mixture was then incubated at 36°C in a water bath for 1 h, dried under a stream of N_2, washed with 400 μl of benzene, and dried. CoA (29 μmol) was dissolved in 400 μl of 0.125 mM NH_4HCO_3 in water adjusted to pH 8.8 with NH_4OH and 0.8 ml of tetrahydrofuran. The mixture was stirred at 36°C in a water bath for 30 min. The reaction was stopped with 20 μl 20% $HClO_4$, and the products were dried under an N_2 gas stream. The residue was dissolved in 100 μl 2% $HClO_4$, and the mixture was lyophilized. The lyophilized G-CoA was dissolved in water, and its concentration was determined by the hydroxamate method (19).

Determination of MCCase and GCCase activities. Cultures of the *P. aeruginosa* PAO1 strain were grown with shaking at 30°C in 50 ml of M9 medium with 0.075% of citronellol or 0.3% of leucine as a carbon source for 48 h. Cells were harvested by centrifugation and washed with 50 ml of 100 mM K_2HPO_4, pH 8.0. Pellets were suspended in 5 ml of the same buffer, disrupted by sonication, and centrifuged for 10 min to 15,000 × *g* at 4°C to eliminate undisrupted cells and cell debris. The protein content was determined by the Bradford method as described previously (22). Purification of MCCase and GCCase from *P. aeruginosa* crude extracts was carried out using avidin-agarose resin (Sigma), with equilibration with phosphate-buffered saline (PBS) (pH 7.4) with 1 mg/ml of biotin; the crude extract was added to the resin and incubated for 2 h at 4°C, the mixture was washed with PBS, and biotinylated proteins were eluted with PBS containing 2 mM biotin. MCCase and GCCase activities in the extracts or in purified fractions were measured by the incorporation of radioactivity from $^{14}CO_2$ into acid-stable, nonvolatile material as previously described (15, 21). The reaction mixture contained 20 mM K_2HPO_4 (pH 8.5), 10 mM $MgCl_2$, cell extract (300 μg of protein) or 40 μg of purified protein, ATP either at 5 mM or in the range of 0 to 40 mM, $NaH^{14}CO_3$ (specific activity, 1.96 GBq/mmol [53 mCi/mmol]; Amersham) either at 10 mM or in the range of 0 to 10 mM, and 3-MC-CoA (Sigma) or *cis*-G-CoA (synthesized as described above) either at 100 μM or in the range of 0 to 100 μM, in a total reaction volume of 100 μl. The reaction was started by the addition of $NaH^{14}CO_3$ (prepared as 1:10 $NaH^{14}CO_3$-$NaHCO_3$ to 10 mM), and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 200 μl of 6 M HCl, the contents of the tube were evaporated to dryness at 90°C, and the residue was suspended in 100 μl of distilled water. Radioactivity was quantified using a liquid scintillation counter (Hewlett-Packard 1600 TR). Nonspecific $^{14}CO_2$ fixation was assayed in the absence of substrate. Specific activity was calculated as dpm of $^{14}CO_2$ fixed/0.53 μmol · min^{-1} · mg^{-1} of protein.

Western blot analysis. *Pseudomonas aeruginosa* cell extract samples containing 100 μg of protein or 10 μg of purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences). MCCase and GCCase α subunits (LiuD and AtuF proteins) were detected by using the avidin-horseradish peroxidase (HRP) conjugate (Bio-Rad) as previously described (1). MCCase and GCCase β subunits (LiuB and AtuC) were detected using polyclonal mouse antibodies. Antibodies were obtained from a polyclonal serum after immunization of mice with LiuB or AtuC recombinant protein expressed from the pTrc-*liuB* and pTrc-*atuC* plasmids and purified from the cell extracts as described above. The polyclonal serum was obtained at the Iowa State University Hybridoma Facility (www.biotech.iastate.edu/service_facilities/hybridoma.html). The membranes were probed using the polyclonal serum, and the anti-mouse–HRP conjugate was used as the second antibody (donkey anti-mouse immunoglobulin G–HRP; Santa Cruz Biotechnology, Inc.) as indicated by the provider. HRP color development was carried out using 4-chloro-1-naphthol (Sigma) and H_2O_2.

RESULTS AND DISCUSSION

Characterization of MCCase and GCCase from *P. aeruginosa* grown on leucine and citronellol. Previous data from our group suggested that MCCase and GCCase show catalytic activity with both MC-CoA and G-CoA substrates, indicating a possible bifunctional role of these enzymes in both the leucine/isovalerate and acyclic terpene catabolic pathways (1). In this work we found that MCCase or GCCase can be coexpressed from cultures of *P. aeruginosa* but that it was difficult to discriminate whether the enzymes showed bifunctionality over both G-CoA and MC-CoA substrates (1). Similar results were found for *P. citronellolis* (9, 15).

To test whether the native properties of MCCase are conserved after heterologous expression of the recombinant enzyme, the MCCase was also purified and characterized as isolated from its native host, *P. aeruginosa.* As indicated in previous reports, MCCase is induced when *P. aeruginosa* grows on leucine as the sole carbon and energy source (1, 16); thus, MCCase was purified by from *P. aeruginosa* cells grown on leucine. With the purified fraction, the optimal pH and temperature for MCCase activity were 8.5 and 37°C, respectively (data not shown). With increasing concentrations of the MC-CoA substrate, a sigmoidal kinetics of MCCase activity was observed (see Fig. S1 in the supplemental material). The kinetic constants for MCCase using MC-CoA as the substrate are a $K_{0.5}$ of 9.2 μM and a V_{max} of 425 nmol/min · mg of protein. Under this condition the MCCase enzyme did not carboxylate the analogous substrate, GC-CoA, indicating that MCCase from *P. aeruginosa* specifically recognizes MC-CoA as its substrate and therefore does not possess GCCase activity (see Fig. S1 in the supplemental material). This is consistent with the behavior of *P. citronellolis* MCCase, which is specific for the MC-CoA substrate and has no detectable GCCase activity (8), as also found in pea leaf and potato mitochondria (2). A difference between the MCCases of *P. aeruginosa* and *P. citronellolis* is the apparent fivefold-higher affinity for the MC-CoA substrate ($K_{0.5}$s of 9.2 μM and 43 μM, respectively). On the other hand, when *P. aeruginosa* was grown on citronellol as the sole carbon source, both the MCCase and GCCase enzymes were expressed (data not shown). Under these conditions MCCase and GCCase were detected and the enzymatic activities displayed similar behavior with both substrates, showing typical sigmoidal behavior with similar kinetic constants (see Fig. S1 in the supplemental material). The kinetic parameters for MCCase and GCCase activities were $K_{0.5}$s of 8.84 and 8.80 μM and V_{max}s of 591 and 627 nmol/min · mg of protein for MC-CoA and G-CoA, respectively. As mentioned above, the purification method used did not differentiate between GCCase and MCCase activities, and therefore it was not possible to elucidate whether GCCase also carboxylates MC-CoA. In addition, these data suggest that GCCase may be nonspecific in its ability to carboxylate G-CoA and MC-CoA or that under the conditions used a subunit exchange mechanism between MCCase and GCCase may possibly contribute to a nonspecific carboxylase enzyme. This behavior could explain why *atuC* and *atuF* mutants are affected in their ability to grow on leucine and why an *atuF* mutant regains the ability to grow on leucine when it is transformed with the *liu* cluster (1). Therefore, we decided to carry out the characterization of both enzymes by expressing them in *E. coli*, a bacterium that does not possess the MCCase and GCCase enzymes.

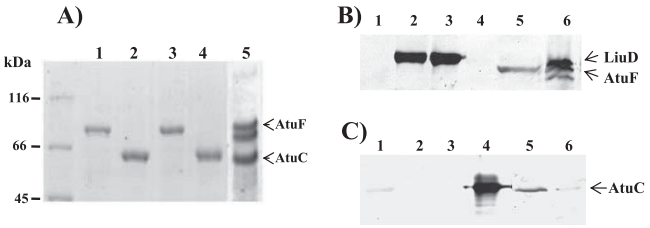

FIG. 2. Electrophoretic analysis of the recombinant heterologous expression of *P. aeruginosa* MCCase and GCCase subunits. (A) Coomassie blue-stained SDS-PAGE analysis of assembled gels of recombinant proteins expressed in *E. coli* and purified using affinity chromatography. Lanes: 1, AtuF-His; 2, AtuC-His; 3, LiuD-His; 4, LiuB-His expressed from pTrcHis-2A vector; and 5, AtuC-His/AtuF-S expressed from pCDFDuet-1 coexpression vector. Molecular mass makers are shown on the left; the protein bands corresponding to AtuC/AtuF are indicated with arrowheads. (B) Western blot analysis of purified recombinant proteins probed with avidin-HRP conjugate to detect biotin-containing proteins. Lanes: 1, LiuB-His; 2, LiuD-His; 3, AtuF-His; 4, AtuC-His; 5, AtuC-His/AtuF-S; and 6, extract from PAO1 culture grown on citronellol. (C) Western blot analysis of purified recombinant proteins probed first with anti-AtuC–His polyclonal antibody and then with anti-mouse–HRP as a secondary antibody. Lanes are the same as in panel B.

Expression of the MCCase and GCCase from *P. aeruginosa* in *E. coli*. The individual enzyme subunits were expressed in *E. coli* and recovered efficiently, obtaining preparations that were >90% pure (Fig. 2A, lanes 1 to 4). In SDS-PAGE the proteins showed relative molecular masses for the LiuD, LiuB, AtuF, and AtuC subunits of 78, 63, 74, and 63 kDa, respectively (Fig. 2A). The expressed and purified LiuB-His and LiuD-His subunits of MCCase did not support enzyme activity. Several attempts to restore the activity of this enzyme using different renaturation conditions were conducted, without success. However, when the purified α and β subunits were combined, denatured, and then renatured as described above, the active MCCase enzyme was recovered. This result shows that incorporation of a His tag does not disturb the function of MCCase, and therefore this recombinant MCCase enzyme was used for the kinetic characterization as described below. In contrast, this strategy was not successful in generating a functional GCCase enzyme. However, a functional GCCase enzyme was successfully reconstituted when the AtuC and AtuF subunit genes

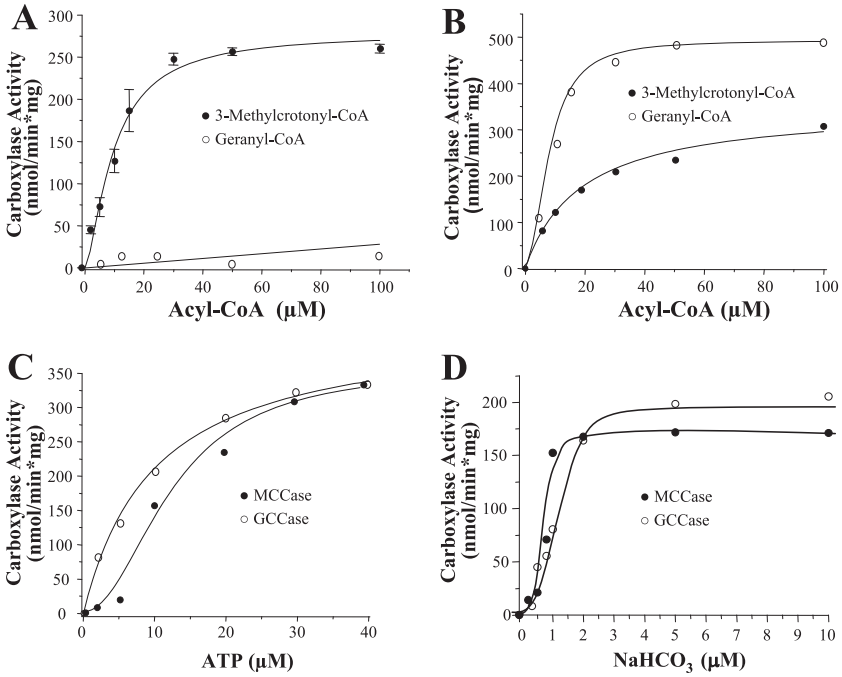

FIG. 3. Kinetic behavior of recombinantly produced *P. aeruginosa* MCCase and GCCase enzymes. (A and B) Carboxylase activities of recombinant LiuB/LiuD (A) and AtuC/AtuF (B) proteins, purified and reconstituted as described in Materials and Methods. (C and D) ATP (C) and bicarbonate (D) concentration dependence of the MCCase and GCCase activities. Data given are the average of three determinations; standard deviations of the given values are shown in panel A, and the averages of two determinations with variations of less than 5% of the given values are shown in panels B, C, and D.

were coexpressed and copurified as described above. The coexpressed AtuF-S protein was found to bind to the AtuC-His subunit and was also copurified (Fig. 2). A similar behavior was observed during the expression of the AccD1-His subunit of acetyl-CoA carboxylase from *Corynebacterium glutamicum* (12) and the acyl-CoA carboxylase from *Streptomyces coelicolor* A3(2) (5).

SDS-PAGE analysis of this purified fraction indicated that it contained three major protein bands (Fig. 2A, lane 5). Western blotting analysis with avidin-HRP conjugate showed that the upper band (74 kDa) was a biotinylated protein, and because it corresponded in molecular mass to the biotinylated subunit of the GCCase purified from *P. aeruginosa* extracts grown on citronellol (Fig. 2B, lanes 5 and 6, respectively), we identified this as the recombinant biotinylated AtuF subunit of the GCCase. Similar Western blot analyses with polyclonal anti-AtuC antiserum identified the lowest band (63 kDa) as the recombinant AtuC subunit, corresponding to the GCCase β subunit; the molecular mass of this band is similar to that of the AtuC subunit purified from *P. aeruginosa* extracts grown on citronellol (Fig. 2C, lanes 5 and 6, respectively). Although only a faint signal was identified in the purified LiuB protein sample with the anti-AtuC serum (Fig. 2C, lane 1), it was clear from the difference in signal intensity that this is a cross-reactivity of the antiserum. These results established that after coexpression of the AtuC-His and AtuF-S proteins in *E. coli*, the GCCase complex was reconstituted, and therefore this complex was used for kinetics characterization. It was found that for both the recombinant MCCase and GCCase enzymes, the optimal pH and temperature were 8.5 and 37°C, respectively (data not shown). These values were identical to those for the native host enzymes and similar to those reported for MCCases from mammalian (18), bacterial (23), and plant (2, 3, 4, 7) sources. Like MCCase from *P. citronellolis*, MCCase and GCCase from *P. aeruginosa* are inactivated by temperatures higher than 50°C (15) (see Fig. S2 in the supplemental material), in contrast to MCCases from pea leaves and potato mitochondria, which are stable above that temperature (2).

Kinetic parameters of recombinant MCCase. The dependence of MCCase activity on substrates was tested at the optimal conditions, pH 8.5 and 37°C. A typical sigmoidal behavior was observed with respect to MC-CoA, while no activity was observed when G-CoA was assayed as the substrate (Fig. 3A). In kinetics calculations the values were adjusted to Hill's equation, showing a correlation coefficient of 0.99 with a Hill's coefficient of 2.3, which suggested that the enzyme has an oligomeric conformation and could provoke cooperative effects of the substrate. The kinetic constants of this enzyme for MC-CoA were a $K_{0.5}$ of 9.8 μM and a V_{max} of 279 nmol/min · mg of protein; these values are in agreement with values obtained with the MCCase purified from of the native host, *P. aeruginosa*. The catalytic efficiencies (V_{max}/K_m) of the two enzyme preparations also were similar (46 and 56, respectively), indicating that the recombinant proteins and the heterologous expression did not affect MCCase functionality, as occurs in other carboxylases (5, 12). On the other hand, the kinetic dependence on the ATP and $NaHCO_3$ substrates showed a sigmoidal response, suggesting an allosteric regulation of MCCase by ATP and $NaHCO_3$ (Fig. 3C and D). The apparent kinetic parameters for ATP were a $K_{0.5}$ of 13 μM and a V_{max} of 356

nmol/min · mg of protein, and those for $NaHCO_3$ were a $K_{0.5}$ of 0.8 μM and a V_{max} of 178 nmol/min · mg of protein. We conclude, therefore, that the MCCase from *P. aeruginosa* is specific for the MC-CoA substrate.

Kinetic parameters of recombinant GCCase. Kinetic parameters for the heterologously coexpressed AtuC/AtuF proteins were also measured at optimal conditions (pH 8.5 and 37°C). Under these conditions, the AtuC/AtuF complex catalyzed both GCCase and MCCase enzymatic activities. In relation to the G-CoA substrate, this enzyme exhibited sigmoidal kinetics, adjusting to Hill's equation with coefficient of 2.2, but in relation to the MC-CoA substrate, it exhibited Michaelis-Menten kinetics (Fig. 3B). These results indicate that the AtuC/AtuF enzyme is able to utilize both G-CoA and MC-CoA as substrates. The kinetic constants with G-CoA were a $K_{0.5}$ of 8.8 μM and a V_{max} of 492 nmol/min · mg of protein, and those with MC-CoA were a K_m of 14 μM and a V_{max} of 308 nmol/min · mg of protein. The catalytic efficiencies for the AtuC/AtuF enzyme were 56 for G-CoA carboxylation and 22 for MC-CoA carboxylation. These results indicate that the AtuC/AtuF enzyme prefers G-CoA over MC-CoA as a substrate, and therefore should be considered a GCCase enzyme. In *P. citronellolis* it has been observed that GCCase is able to carboxylate 5 to 15 different acyl-CoA substrates, including MC-CoA (8). An interesting fact is that the plant GCCase shows a strict substrate preference, carboxylating G-CoA but not MC-CoA (13). This finding suggests that in *P. aeruginosa* GCCase may play a bifunctional role during its participation in both the acyclic terpene and the leucine catabolic pathways. Using G-CoA as the carboxylation substrate, the dependence of enzymatic activity on ATP and $NaHCO_3$ was also tested. In relation to ATP a typical Michaelis-Menten kinetics was observed (Fig. 3C), whereas with $NaHCO_3$ a sigmoidal kinetics was observed (Fig. 3D). The kinetic parameters of GCCase for ATP were a K_m of 10 μM and a V_{max} of 423 nmol/min · mg of protein, and those for $NaHCO_3$ were a $K_{0.5}$ of 1.2 μM and a V_{max} of 210 nmol/min · mg of protein.

The kinetic parameters of both MCCase and GCCase with the cosubstrates ATP and $NaHCO_3$ followed similar tendencies, as could be expected because they have common cosubstrates.

This is the first report showing that MCCase and GCCase display sigmoidal kinetic behavior for their substrates and cosubstrates. These results are interesting because most of the MCCases characterized to date show typical Michaelis-Menten kinetics against their substrates. A sigmoidal kinetic behavior would indicate that the active site is modified by the binding of each substrate, increasing the affinity for the next one, and/or that the binding of substrate promote the oligomerization of the enzyme subunits, favoring their activity. A precedent that supports this finding may be found with pyruvate carboxylase and the MCCases from pea leaf and potato mitochondria, in which the domain arrangement provides a mechanism for allosteric activation (24) and sigmoidal behavior with respect to Mg^{2+} variations (2, 3).

In conclusion, our results indicate that MCCase, encoded by *liuB*/*liuD* genes, specifically recognizes MC-CoA as its substrate, and the bifunctionality of the GCCase suggests that it may supplant the MCCase function in leucine catabolism and therefore that both the MCCase and GCCase enzymes might play important roles in the catabolic pathways for leucine as well as for acyclic terpenes.

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IX. DISCUSION GENERAL

Con la utilización de mutantes individuales por transposición se logró establecer que los operones *gny* (*liu*) y *atu* están involucrados en el catabolismo de leucina, isovalerato y citronelol; tal resultado fue corroborado por análisis de complementación de las mutantes, así como también por análisis de secuencias en bancos de datos que indicaron que PA2888 y PA2014 presentaban similitud con la subunidad β, y PA2891 y PA2012 presentan similitud con la subunidad α de enzimas acil-CoA carboxilasas, así como un 63% y un 64% de similitud entre ellas, respectivamente. En AtuF y LiuD se encontraron las secuencias de unión al ATP (GXGXXG), la de fijación de CO_2 (RDCS), la secuencia del sitio catalítico de carboxilasas dependientes de biotina (EMNTR) y el dominio de biotinilación (AMKM). En AtuC y LiuB se encontraron los dominios de unión al acil-CoA y a la carboxibiotina (Song *et al*., 1994). Todo lo anterior sugiere que estos cuatro genes codifican para las subunidades α y β de enzimas acil-CoA carboxilasas.

El ORF PA2889 (AtuD) mostró similitud con enzimas acil-CoA deshidrogenasas y un 34% de identidad con PA2015 (LiuA), lo que sugiere que LiuA también es una deshidrogenasa. El ORF PA2890 (AtuE) resultó ser similar a enoil-CoA hidratasas/isomerasas, al igual que LiuC (ORF PA2013), y entre ellas hay una identidad de 38%. El ORF PA2893 (AtuH) presentó similitud con acil-CoA sintetasas de cadena larga, lo que sugiere que este ORF esta asociado al operón *atu*, sin embargo, la mutante interrumpida en este gen creció de manera normal tanto en citronelol como en leucina como fuente de carbono, por lo que se descarta su participación.

Al realizar un análisis de secuencia de aminoácidos de las proteínas AtuC, AtuD, AtuE y AtuF, entre varias especies de *Pseudomonas*, se encontraron probables homólogos en los genomas de *P. putida* KT2440, *P. fluorescens* PfO-1, *P. syringae* pv. tomato D3000 y *P. syringae* pv. syringae B728a, en las que solamente se encontró un homólogo, mientras que *P. aeruginosa* PAO1 presenta dos; esto hace más evidente su elevada capacidad metabólica.

De manera muy interesante, se observó, que los productos del operón *liu* son más parecidos a los productos del operón *liu* de otras *Pseudomonas* (de 77.2 a 79.5 %) que a los productos del operón *atu* de la misma bacteria (41.5 %). Por otro lado, en el operón *atu* no se

encontró ningún homólogo de *liuE*, lo que puede sugerir una función dual de LiuE, tanto en el catabolismo de isoprenoides acíclicos como en el de leucina.

La realización de Western blots del extracto crudo de *P. aeruginosa* PAO1 crecida en glucosa presentó la expresión de dos proteínas biotiniladas, una de 22 y la otra de 62 kDa; cuando se crece en leucina se presenta, además, una proteína de 73 kDa, que corresponde a la subunidad α de la enzima MCCasa, misma que no se observó en la mutante en *liuD*; cuando se utilizó citronelol como fuente de carbono, aparece una proteína de 70 kDa, que corresponde a la subunidad α de la enzima GCCasa, que no se observa en la mutante interrumpida en *atuF*. Así se observa que solamente el citronelol es capaz de inducir la expresión de las dos carboxilasas.

Las clonas con la GCCasa no funcional crecieron en leucina, aunque de manera escasa, y ninguna de las mutantes individuales de MCCasa y GCCasa mostraran crecimiento en citronelol como fuente de carbono y energía, además, al complementar a la mutante interrumpida en *atuF* con el operón *liu* o con el operón *atu*, ésta recuperó su capacidad de crecimiento en citronelol. Lo anterior sugiere que probablemente estas enzimas puedan carboxilar cada una al sustrato de la otra.

Inicialmente se observó que en cultivos de *P. aeruginosa* PAO1 crecida en leucina como fuente de carbono y energía, únicamente se expresa la MCCasa, mientras que utilizando citronelol se expresan tanto la MCCasa como la GCCasa (Hoschle *et al*., 2005; Aguilar *et al*., 2006; Forster-Fromme *et al*., 2006). En ensayos de actividad de MCCasa, realizados con el extracto crudo de *P. aeruginosa* PAO1 crecido en citronelol, se observó un incremento de ~3 veces en la actividad, con respecto al realizado con el extracto crudo de la bacteria crecida en leucina. Estos resultados refuerzan la probable participación dual de las dos enzimas.

Para confirmar la actividad dual de las enzimas GCCasa y MCCasa se realizó la caracterización cinética de ambas. Ya se ha descrito la purificación de MCCasa a partir del extracto crudo de *P. aeruginosa* cultivada en leucina (Fall, 1981), y también se puede utilizar avidina como ligando para proteínas biotiniladas. De esta manera se purificó la MCCasa la que al realizar su caracterización bioquímica se obtuvieron los siguientes datos:

La MCCasa tiene un pH y temperatura óptimos de 8.5 y 37°C, respectivamente. Estos valores son similares a los reportados para la enzima MCCasa de chícharo (Baldet *et al*., 1992), de papa (Alban *et al*., 1993), la de *Achromobacter* (Schiele y Lyne, 1981) y la de mamíferos (Lau *et al*., 1980). Las enzimas MCCasa y GCCasa de *P. aeruginosa* son inactivadas por temperaturas mayores a los 50°C, al igual que la MCCasa de *P. citronellolis* (Hector y Fall, 1976); mientras que la enzima de chícharo y la de mitocondria de papa soportan más de 50 °C sin sufrir desnaturalización (Alban *et al*., 1993). La cinética mostrada por MCCasa fue una típica curva sigmoidal con $K_{0.5}$ de 9.2 μM para MC-CoA y V_{max} de 425 nmol/min·mg de proteína, mientras que la eficiencia catalítica fue de 46.2. Utilizando GC-CoA como sustrato no se observó actividad de carboxilasa, lo que sugiere que la MCCasa de *P. aeruginosa*, purificada a partir de cultivos realizados en leucina como fuente de carbono, reconoce específicamente al MC-CoA como sustrato. Este resultado coincide con el reportado por Fall (1981) para la enzima de *P. citronellolis* y por Alban y col. (1993) para las MCCasas de chícharo y de mitocondria de papa.

Cuando *P. aeruginosa* es crecida en citronelol como única fuente de carbono y energía se expresan ambas enzimas, MCCasa y GCCasa. Con los procedimientos descritos las dos enzimas copurifican, lo que no permite discriminar entre la actividad sobre G-CoA y la actividad sobre MC-CoA. La caracterización de la enzima GCCasa se realizó por medio de un sistema de expresión heteróloga en *E. coli*. Asímismo, se realizó también la expresión heteróloga de la MCCasa con propósitos comparativos.

Los péptidos heterólogos fueron purificados y combinados físicamente para constituir la enzima (la subunidad α con la subunidad β correspondiente). Siguiendo este procedimiento, ninguna de las dos enzimas reconstituidas mostró actividad, debido a lo cual se realizó un proceso de desnaturalización-renaturalización de los péptidos, similar al reportado por Gago y col. (2006). Con este nuevo procedimiento, no se observó actividad de ninguna de las dos enzimas. A continuación se cambió el sulfato de amonio por urea 6 M y al final de este procedimiento la enzima MCCasa se obtuvo en forma activa, no así la GCCasa. Este fenómeno puede ser debido al plegamiento natural del péptido y que al entrar en contacto con la otra subunidad no puede realizar las interacciones proteína-proteína necesarias para lograr

obtener una enzima funcional; otra posibilidad es la formación de cuerpos de inclusión generados por la gran cantidad de proteína expresada.

Para obtener la enzima GCCasa activa, la estrategia utilizada fue empleando un vector de expresión dual en el cual se pueden insertar los genes de las dos subunidades y estas son expresadas al mismo tiempo. El vector empleado fue pCDFDuet-1 (Novagen) en la construcción obtenida, pCFC3-atuFC, que porta a los genes *atuFC*, solamente al péptido AtuC le es adicionado el hexapéptido de histidinas, por lo que al purificarse por cromatografía de afinidad, el péptido AtuF es copurificado. Con la utilización de este vector la enzima GCCasa se obtuvo de manera activa. Al realizar un corrimiento electroforético de la enzima purificada en SDS-PAGE, se observó la presencia de tres bandas, dos de ellas corresponden a las subunidades de la enzima, y una adicional de aproximadamente 70 kDa que al realizar el Western blot con avidina-HRP no es detectada, por lo que no corresponde a una proteína biotinilada, además de ser de menor tamaño que la subunidad α (biotinilada), pero mayor que la subunidad β. Hasta el momento desconocemos de qué proteína pudiera tratarse. Con la utilización de ambos vectores, la purificación de la proteína en base a los corrimientos electroforéticos en geles de poliacrilamida, se observa ser mayor al 95%, grado adecuado para nuestros propósitos.

La enzima MCCasa recombinante mostró una cinética y condiciones óptimas similares a las presentadas por la enzima purificada de *P. aeruginosa*. Igualmente, no mostró actividad catalítica sobre G-CoA, lo que sugiere que la enzima es específica. Por otro lado, la enzima GCCasa recombinante mostró actividad tanto con G-CoA como con MC-CoA, y las constantes cinéticas fueron $K_{0.5}$ de 8.8 μM para G-CoA y K_m de 14 μM para MC-CoA; V_{max} de 492 y 308 nmol/min.mg de proteína, y eficiencias catalíticas de 56 y 22, para G-CoA y MC-CoA, respectivamente. Estos resultados sugieren que el G-CoA es preferido como sustrato por esta enzima; también se observó la inespecificidad o bifuncionalidad de la enzima GCCasa, la cual podría tener injerencia tanto en la degradación de terpenos acíclicos como en el catabolismo de leucina.

La eficiencia catalítica de la GCCasa (56) de *P. aeruginosa* es mucho más elevada que la de *Zea mays* (2.3) (Guan *et al*., 1999). La eficiencia catalítica de la MCCasa de *P. aeruginosa* es similar a la de mitocondria de hoja de chícharo (Alban *et al*., 1993). Lo anterior

señala la existencia de diferencias funcionales y estructurales entre las enzimas de estas dos fuentes. Otra diferencia se observa en la masa, ya que en *P. aeruginosa* y en *P. citronellolis* la subunidad α tiene una masa aproximada de 73 kDa, mientras que en plantas es de 122 kDa.

Se ha reportado que el arreglo tridimensional en algunos miembros de esta familia de enzimas dependientes de biotina es $\alpha_4\beta_4$, por lo que el comportamiento sigmoidal de la cinética con algunos sustratos podría indicar que el sitio activo es modificado por la unión de tal sustrato; esto podría incrementar la afinidad por el próximo sustrato. Esto también podría sugerir que la unión del sustrato promueve la oligomerización de las subunidades de la enzima, favoreciendo la actividad.

Este comportamiento sigmoidal de la GCCasa se ha relacionado con el que muestra la piruvato carboxilasa, en la que el arreglo de los dominios facilita un mecanismo de activación alostérica (Martin *et al*., 2007). Los resultados obtenidos también son interesantes porque la mayoría de las MCCasas reportadas presentan comportamientos cinéticos del tipo de Michaelis-Menten, aunque las MCCasas de hoja de chícharo y de mitocondria de papa presentan un comportamiento sigmoidal al variar las concentraciones de Mg^{2+} (Baldet *et al*., 1992; Alban *et al*., 1993).

X. CONCLUSION

Los genes *atuCF* y *liuBD* del cromosoma de *Pseudomonas aeruginosa* PAO1 codifican las subunidades β y α de las enzimas geranil-CoA carboxilasa y 3-metilcrotonil-CoA carboxilasa, respectivamente. La actividad de la enzima MCCasa heteróloga resultó estar restringida a la ruta degradativa de leucina, al mostrar carboxilación de MC-CoA y no de G-CoA. Mientras que la GCCasa heteróloga, producida en *E. coli* que porta a los genes de la enzima en un vector de expresión dual, al presentar actividad con ambos sustratos, sugiere que, además de actuar en la ruta degradativa de isoprenoides acíclicos, también participa en la ruta degradativa de leucina.

XI. PERSPECTIVAS

Para continuar este trabajo se proponen los siguientes puntos:

1. Determinar la posible carboxilación que pudieran presentar de otros compuestos de estructura similar.
2. Complementación de las subunidades, esto es, analizar si la subunidad α de la MCCasa puede formar una enzima activa con la subunidad β de la GCCasa, y viceversa.
3. Resolver la estructura de estas enzimas por cristalografía de rayos X o por RMN.

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