

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-068859- -00009-0000

Fecha: 2012-11-21 15:09:37

Dep. 2020 DIR.NUEVASCR

Tra. 2 PATENTES

Eve: 1 REGDEPOSITO

Act. 523 RESPUESTA

Folios: 50

Clarke, Modet & Cº

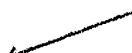
COLOMBIA

P-1335

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **08.068.859** 
Solicitud de patente a nombre de **UNIVERSIDAD TECNICA
FEDERICO SANTA MARIA**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada de acuerdo con la sustitución del 17 de agosto de 2010, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, de la sociedad **UNIVERSIDAD TECNICA FEDERICO SANTA MARIA** domiciliada en Valparaiso, Chile, solicitante de la patente de la referencia, en respuesta al oficio No. 12154 notificado por fijación en lista el 13 de julio de 2012, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio que reemplaza el antiguo juego de reivindicaciones y que consta de 9 reivindicaciones dirigidas al producto para la biorremediación de ambientes contaminados de acuerdo con la invención y al método para la biorremediación.

En este sentido nos permitimos indicar que cuales fueron las modificaciones realizadas en las reivindicaciones:

Se ha cancelado la cláusula 1, dirigida a una nueva cepa bacteriana, como fue originalmente presentada, al estar excluida de patentabilidad.

Se han fusionado las anteriores cláusulas 2 y 3, dirigidas a un producto para la biorremediación, conformando una nueva reivindicación 1. Consecuentemente la original cláusula 3 fue cancelada del Pliego.

Además, para mayor claridad, la nueva reivindicación 1, detalla el número y fecha de depósito de la cepa bacteriana antes recitado en la original cláusula 1; y, donde también, se han precisado las concentraciones de la cepa bacteriana en virtud de los medios ahora claramente definidos. En particular se aprecia que la solución tampón es fosfato de sodio 5-100 mM (pH 7), y si bien los medios de cultivo se describen con detalle en el texto (Tabla 3), se limita el medio de cultivo a un medio mínimo con fuente de carbono (succinato) en un rango de 0,5 a 30 mM.

Por otra parte, las anteriores cláusulas 4 y 5, ahora reenumeradas 2 y 3; sólo fueron modificadas en su dependencia para guardar consistencia con las enmiendas a las reivindicaciones precedentes. Por otra parte, la cláusula 3 ahora precisa que el alginato de sodio usado puede variar en un rango de 0,5-5%. Sustento para esta inclusión se puede encontrar en la descripción de la solicitud, donde se menciona (página 25, renglones 8-11 y figura 7) “El proceso de encapsulación con alginato de sodio 1% permite el crecimiento de *Pseudomonas* sp. MHP41, no afectando negativamente su viabilidad”.

También se han fusionado las cláusulas 6 y 8 originales, dirigidas a un método para la bioremediación de un ambiente contaminado con s-triazinas, para formar una nueva reivindicación 4. Consecuentemente la original cláusula 8 fue cancelada del Pliego. De esta manera queda claro que el método que se pretende proteger no corresponde tan sólo al uso del producto antes reivindicado. Por el contrario, el método precisa que se debe agregar el producto, luego esperar por un determinado periodo de tiempo, y ahora, claramente se establecen las concentraciones de la bacteria en el ambiente contaminado, que es habitualmente suelo.

Por otra parte, las anteriores cláusulas 7, y 9-12, ahora reenumeradas 5 y 6-9, respectivamente; sólo fueron modificadas en su dependencia para guardar consistencia con las enmiendas a las reivindicaciones precedentes.

Sobre este particular, nos permitimos manifestar que las modificaciones anteriormente mencionadas no amplían el alcance original de la invención y se dan con el ánimo de subsanar las objeciones de claridad, establecidas por el señor examinador en su concepto técnico del oficio No. 12154.

Teniendo en cuenta el concepto técnico emitido por el Señor Superintendente Delegado para la propiedad intelectual GianCarlo Marcenaro Jiménez a la doctora Ana Maria Castro Wey, que da alcance a las comunicaciones No. 09-001524 y 09-012566, no se presenta una tasa adicional, toda vez que la solicitud fue presentada con anterioridad al 1 de enero de 2009.

2. No es una invención

Se ha cancelado la cláusula 1, dirigida a una nueva cepa bacteriana, como fue originalmente presentada, al estar excluida de patentabilidad.

3. Reivindicaciones relativas al uso

En relación con las objeciones planteadas por el señor examinador, atentamente nos permitimos decir que con el nuevo capítulo reivindicatorio se superan dichas objeciones, toda vez que el nuevo pliego reivindicatorio no contiene esta clase de cláusulas.

4. Respuestas a los puntos de la Acción Oficial

4.1 Punto 3. Objeto de la Invención

El solicitante acepta la propuesta del problema técnico planteado por el examinador. Sin embargo, es deseo aclarar que la solución al dicho problema técnico planteado proveer un producto nuevo e inventivo para la biorremediación de ambientes contaminados con compuestos s-triazinas, no sólo comprende un inóculo bacteriano de la cepa *Pseudomonas* sp MHP41, con depósito bajo el acceso NRRL B-30908; sino que además incorpora un medio de cultivo definido (succinato 0,5-30 mM), o un medio buffer definido (5-100 mM fosfato de sodio, pH 7), o alginato de sodio (0,5-5%). En este sentido quedará claro que el producto que se desea proteger viene a ser una composición de otros elementos adicionales, además de la cepa bacteriana antes indicada.

4.2 Punto 4. No es una Invención [Art 15 D 486 literales (b)]

Párrafo 1. El examinador plantea “El objeto de la reivindicación 1 no se considera una invención, de acuerdo con el Artículo citado, ya que corresponde al aislamiento de un microorganismo existente en la naturaleza y que no ha sido modificado por el solicitante”.

El solicitante acepta que la reivindicación 1, que se refiere a una bacteria aislada del suelo y que no ha sido modificada genéticamente por los inventores, no se considere una invención en Colombia. De acuerdo a esto, dicha cláusula 1 original ha sido eliminada del Pliego de Reivindicaciones pendientes.

Párrafo 2. El examinador también plantea “El objeto de la reivindicaciones 2-5 no se considera una invención, de acuerdo con el Art citado, ya que si bien se refiere a un producto, tanto la descripción como las reivindicaciones definen el

producto como un inóculo de la bacteria, en el cual el solicitante no ha aportado a la composición ningún excipiente, por lo que el producto se reduce a el aislamiento de la bacteria de interés”.

Sobre este particular, es deseo del solicitante manifestar que se encuentra en respetuoso desacuerdo respecto a dicha opinión. En particular, tal como se constató antes, quisiéramos indicar al examinador las reivindicaciones 2-5 de la presente solicitud de patente se refieren a productos que sí constituyen una invención. Dicho productos, si bien se basan en un inóculo bacteriano; este no es el componente único de tal producto.

En particular la nueva cláusula 1 se refiere al producto para la bioremediación que contiene un inóculo de la bacteria *Pseudomonas* sp. cepa MHP41 NRRL B-30908 y un medio de cultivo, una solución tampón o alginato de sodio, que permiten mantener la actividad degradadora de la cepa bacteriana. De esta manera tanto el medio de cultivo, el medio tampón y/o el alginato de sodio pueden tenerse como componentes adicionales y necesarios en el producto que permite y favorece la actividad degradadora de la bacteria. La preparación del producto que contiene la bacteria con el medio de cultivo, el medio tampón y alginato de sodio son descritos en la descripción de esta solicitud.

Por su parte, la nueva cláusula 2 se refiere al producto para la bioremediación que contiene células liofilizadas de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908 en un medio protector complejo estéril. El medio protector complejo estéril puede ser definido como un excipiente que favorecen la viabilidad y la actividad degradadora de la bacteria en el tiempo. La preparación del producto que contiene la bacteria con el medio protector estéril se describe en la descripción de esta solicitud.

Además, la nueva cláusula 3 se refiere al producto para la bioremediación que contiene células encapsuladas en alginato de sodio de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908. Células encapsuladas en alginato de sodio poseen una mayor viabilidad celular y preservan la actividad degradadora de *s*-trazinas de *Pseudomonas* sp. MHP41 NRRL B-30908. La preparación del producto que contiene células encapsuladas en alginato de sodio de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908 también se describe en la descripción de esta solicitud. Por otra parte, en el estado del arte se considera que células encapsuladas en alginato de sodio efectivamente constituyen un producto.

Cabe mencionar, que el encapsulamiento con alginato de sodio protege a bacterias de la invención, tanto de la predación por protozoos, como de posibles tóxicos presentes en la adaptación al suelo, o de condiciones extremas como por ejemplo resequedad, altas concentraciones de sales u otros

que puedan estar presentes en el suelo a tratar. Evidencia de esto se puede encontrar en la publicación de Morgante et al (Ver documento D6, adjunto) publicada en su versión online el pasado 2-noviembre-2009. Los medios mínimos y las soluciones tampones protegidos bajo el alcance de la nueva reivindicación 1 enmendada, permiten mantener una alta actividad de los enzimas catabólicos, dado que sus genes se expresan en estas condiciones. La determinación de las concentraciones óptimas de estos medios de cultivo, solución tampón o alginato de sodio no puede tenerse por obvia, ya que fueron determinadas específicamente para la cepa de la invención. Además no puede establecerse *a priori* que ciertas concentraciones de medios de cultivo, soluciones tampón o alginato de sodio para un tipo de bacteria, sean directamente aplicables a otras.

4.3 Punto 5. Reivindicaciones relativas a un uso o a un segundo uso [Art 14 y 21 D 486]

El examinador plantea en este punto “El uso de las de la reivindicaciones 6 a 12 no es patentable, de acuerdo con el Art 14, ya que dichas reivindicaciones se refieren a métodos que sólo constituyen el dispersar en inóculo de *Pseudomonas* sp. MHP41 NRRL B-30908 en un ambiente contaminado. Además, el resultado del método corresponde a un proceso esencialmente biológico, ya que *Pseudomonas* sp. MHP41 NRRL B-30908 es un microorganismo aislado, sobre el cual el solicitante no realizó ninguna modificación y que tiene dentro de sus actividad natural la degradación de s-triazinas”.

Nuevamente, el solicitante manifiesta que se encuentra en respetuoso desacuerdo respecto a dicha opinión. En particular, la materia bajo el alcance antes cubierto por las cláusulas 6-12 (ahora correspondientes a las reivindicación 4-9) de la presente solicitud se refieren a métodos de aplicación de un producto, con pasos y condiciones definidas, y por tanto constituyen materia patentable.

En particular la cláusula principal de método (nº 4) desea proteger corresponde a la aplicación de un producto, en base a un inóculo, a un suelo contaminado, en intervalos de tiempo (incubar por 1 semana a 12 meses) determinados, como corresponde a un método. Es más, en razón de las enmiendas realizadas, la concentración del inóculo del producto en dicho suelo ahora es recitada en dicha reivindicación 4. Por consiguiente, el método recitado en la nueva reivindicación 4 enmendada no constituye un método esencialmente biológico ni tampoco el simple uso del inóculo bacteriano. Luego, en las reivindicaciones dependientes (nº 5-9) enmendadas se establecen limitaciones respecto al tiempo de incubación, y sobre los contaminantes presentes en el suelo contaminado. En otras palabras, las reivindicaciones de método claramente

involucran, dentro de su alcance, la determinación de parámetros de aplicación relevantes (desarrollo por parte de los inventores) pues tales demuestran que el procedimiento ha sido optimizado, y en consecuencia ya no trata del uso de un método esencialmente biológico. En el estado del arte, es conocido que el éxito de un método de bioremediación depende de múltiples factores bióticos y abióticos, y su eficacia debe ser demostrada experimentalmente. Los métodos reivindicados se describen en la descripción de esta solicitud.

Cabe recalcar que los experimentos de bioremediación diseñados y realizados en la presente solicitud, corresponde a ensayos con suelos “reales” y en suelos esterilizados. Dichos suelos “reales” del campo utilizados se encuentran efectivamente contaminados. Tal como se define en la presente solicitud, “Los “microcosmos” representa a un cierto volumen de suelo dispuesto en un recipiente, cuyas variables relevantes tales como la humedad, la temperatura y la presencia o ausencia de microorganismos y/o contaminantes orgánicos son conocidas y controladas.” En este sentido, el uso de microcosmos es de amplio conocimiento en el área técnica, y no debe quedar duda que los suelos donde se ensayó el producto de la invención, que han sido estandarizados para evidenciar sus efectos, son suelos contaminados “reales”. Específicamente, la esterilización buscaba evitar la posible degradación de simazina por otro microorganismo presente naturalmente en el suelo. Una evidencia del uso de suelo “real”, se puede encontrar en la Descripción, página 8, donde se divulga: “La figura 9 muestra la cinética de degradación de *Pseudomonas* sp. MHP41 en experimentos de biorremediación en microcosmos de suelos con historial de aplicación de simazina...” Es decir, el suelo utilizado en el ejemplo se obtuvo de una zona agraria donde se había utilizado simazina en más de una oportunidad.

4.4 Comentarios Adicionales

El examinador finaliza en la acción oficial con “A pesar de que el capítulo reivindicativo corresponde a materia no patentable, se aclara al solicitante que en el estado de la técnica se encontraron los siguientes documentos que anticipan la presente solicitud.

[se incluye una tabla con los detalles de los documentos: D1 WO03025627; D2 Appl. Environ. Microbiol 70(4): 1907-1912]

D1 divulga un producto de bioremediación de ambientes contaminados con simazina, que incluye un inóculo de *Pseudomonas* sp. y alginato de sodio, dicho inóculo puede estar o no liofilizado en un complejo protector estéril.

D2 divulga una cepa de *Pseudomonas* sp. ADP que es capaz de degradar atrazina como única fuente de nitrógeno”.

En relación con estos argumentos, respetuosamente nos permitimos decir que los inventores de la presente solicitud no se encuentran de acuerdo con los mismos. En particular, se acompañan los siguientes argumentos para reafirmar que la solicitud de patente presenta novedad, y no es anticipada D1 ni tampoco por D2.

El documento D1 corresponde a la patente WO 03025627 de Reardon y Das que reivindica un biosensor para la detección de contaminantes orgánicos halogenados, que podría contener enzimas y células completas de que degradan s-triazinas como las células de bacterias tales como *Pseudomonas* sp. ADP, *Ralstonia* sp. M91-3, *Clavibacter michiganese* sp. ATZ1, *Agrobacterium* sp. J14a, *Alcaligenes* sp. SG1, *Rhodococcus* spp. NI86/21 y TE1, *Pseudomonas* spp. 192 y 194, y *Streptomyces* sp. PS1/5. Reardon y Das reivindican un biosensor, pero no se reivindica la aplicación de las bacterias para la bioremediación. Mientras que la presente solicitud de patente en Colombia reivindica un producto para la bioremediación de suelos contaminados y aguas. Así, los productos de ambas solicitudes de patentes tienen aplicaciones diferentes.

Por otro lado, *Pseudomonas* sp. cepa MHP41 es genéticamente diferente que las bacterias indicadas en el documento de la patente WO03025627. *Pseudomonas* sp. MHP41 cepa pertenece a un grupo filogenético diferente que las cepas de los géneros *Ralstonia*, *Clavibacter*, *Agrobacterium*, *Alcaligenes*, *Rhodococcus* y *Streptomyces*. La cepa *Pseudomonas* sp. MHP41 es genéticamente diferente que la bacteria degradadora de atrazina *Pseudomonas* sp. ADP y *Pseudomonas* spp. cepas 192 y 194. *Pseudomonas* sp. MHP41 se diferencia en su perfil molecular ARDRA del gen RNAr 16S y en su secuencia del gen RNAr 16S de *Pseudomonas* sp. ADP. Esto se describe en la solicitud de patente: perfil molecular ARDRA del gen RNAr 16S (Figura 5) y en su secuencia del gen RNAr 16S (en el texto de la sección "Descripción de la cepa *Pseudomonas* sp. MHP4"). Las diferencias moleculares de ambas cepas MHP41 y ADP permiten llegar a la conclusión que son especies diferentes. Asimismo, *Pseudomonas* sp. cepa MHP41 está estrechamente relacionada con *P. nitroreducens* (Ver Hernández et al. 2008, documento D3 adjunto). Por lo tanto, *Pseudomonas* sp. MHP41 es una cepa de una especie diferente que las cepas 192, 194 y ADP. En resumen, *Pseudomonas* sp. MHP41 es una cepa genéticamente diferente que las bacterias incluidas en la patente WO03025627 de Reardon y Das. De esta manera *Pseudomonas* sp. MHP41 es una cepa bacteriana que presenta novedad y no es anticipada por el documento D1.

El documento D2 se refiere a la bacteria *Pseudomonas* sp. cepa ADP y su degradación simultanea de atrazina y fenol. *Pseudomonas* sp. cepa MHP41 se diferencia y presenta ventajas frente a dicha cepa ADP. *Pseudomonas* sp. MHP41

se diferencia en su perfil molecular ARDRA del gen RNAr 16S y en su secuencia del gen RNAr 16S de *Pseudomonas* sp. ADP, como se indicó anteriormente. Esto se describe en la solicitud de patente: perfil molecular ARDRA del gen RNAr 16S (Figura 5) y en su secuencia del gen RNAr 16S (en el texto de la sección “Descripción de la cepa *Pseudomonas* sp. MHP41”). Las diferencias moleculares de ambas cepas MHP41 y ADP permiten llegar a la conclusión que son especies diferentes.

Se presentan algunas ventajas de la aplicación para procesos de bioremediación de *Pseudomonas* sp. cepa MHP41 sobre *Pseudomonas* sp. cepa ADP. La bioaumentación de suelos agrícolas del valle de Aconcagua mediante el producto que comprende la cepa MHP41, cepa que presenta novedad, y que por ende, provee novedad al producto que lo comprende; permitió una remoción casi completa del herbicida simazina (100 ppm) a los 28 días. Esto se describe en la solicitud de patente (en texto de la descripción y Figura 8). De esta manera se demuestra experimentalmente la capacidad del producto de la invención de bioremediar suelos contaminados con el herbicida. En contraste, la bioremediación de simazina en suelos agrícolas del valle de Aconcagua mediante *Pseudomonas* sp. ADP no fue exitosa (Morán *et al.*, 2006, documento D4 adjunto). Como indican los autores del estudio, en suelos agrícolas del valle de Aconcagua bioaumentados con la cepa ADP no se observó un incremento de la remoción de simazina (40 ppm) respecto al suelo control no inoculado a las 28 días (Ver documento D4).

La bacteria *Pseudomonas* sp. cepa MHP41 mantiene una importante capacidad de degradación de *s*-triazinas en presencia de otras fuentes de nitrógeno como nitrato, urea y amonio. Esto se describe en la solicitud de patente (en texto de la descripción y Tabla 4). En contraste, *Pseudomonas* sp. ADP, pierde su capacidad de degradar atrazina al ser cultivada en presencia de amonio, urea o nitrato (Ver documento D5 adjunto), lo que limita sus aplicaciones en bioremediación de suelos. Por consiguiente, el producto de la invención que comprende la cepa MHP41 puede ser un excelente biocatalizador para ser empleado en estrategias de bioremediación en suelos, principalmente de uso agrícola, los que están rutinariamente expuestos a procesos de fertilización con fuentes exógenas de nitrógeno como urea, nitrato y amonio.

Hacemos notar que la presente solicitud, en reivindicaciones similares a las ahora enmendadas en la presente solicitud, obtuvo la aprobación y concesión en Canadá en la patente CA 2636856 y en Estados Unidos (por publicar); confirmándose la novedad y altura inventiva no solo de la bacteria *Pseudomonas* sp. cepa MHP41 sino que además del producto para bioremediación que la comprende.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *“habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares”* y *“(.) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

Señor Superintendente,



DILIA MARIA RODRIGUEZ D'ALEMAN
C.C. 41.654.882 de Bogotá
T.P. 20824 del C.S.J.
notificaciones@clarkemodet.com.co

PLIEGO DE REIVINDICACIONES

1. Un producto para la biorremediación de ambientes contaminados con compuestos s-triazinas, CARACTERIZADO porque comprende un inóculo bacteriano de la cepa *Pseudomonas* sp. MHP41, depositada bajo el número de acceso NRRL B-30908 con fecha 17 de marzo de 2006, en una concentración desde 1×10^3 UFC/ml hasta 1×10^{15} UFC/ml de medio de cultivo con 0,5-30 mM de succinato (fuente de carbono) o de solución tampón con 5-100 mM de fosfato de sodio (pH 7), o desde 1×10^5 UFC/g hasta 1×10^{12} UFC/g de alginato de sodio.

2. El producto para la biorremediación de acuerdo a la reivindicación 1, CARACTERIZADO porque dicho inóculo contiene células liofilizadas de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908 en un medio protector complejo estéril.

3. El producto para la biorremediación de acuerdo a la reivindicación 1, CARACTERIZADO porque dicho inóculo contiene células encapsuladas en alginato de sodio (0,5-5%) de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908.

4. Un método para la biorremediación de un ambiente contaminado con s-triazinas, CARACTERIZADO porque dicho método comprende:

a) agregar un producto de acuerdo con cualquiera de las reivindicaciones 1 a 3 a dicho ambiente contaminado con s-triazinas, y

b) incubar dicho producto que contiene el inóculo de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908 en este ambiente contaminado, y dejar que este actúe durante un periodo de tiempo suficiente, desde al menos 1 semana hasta alrededor de 12 meses, para permitir la degradación de dicho compuesto s-triazina contenido en dicho ambiente donde la concentración de la cepa *Pseudomonas* sp. MHP41 NRRL B-30908 en dicho ambiente contaminado, el que es habitualmente suelo, es desde alrededor de 1×10^2 UFC/g de suelo hasta alrededor de 1×10^{12} UFC/g de suelo.

5. Un método para la biorremediación de un ambiente contaminado con s-triazinas de acuerdo a la reivindicación 4, CARACTERIZADO porque dicho periodo de tiempo comprende un periodo desde 1 semana hasta 4 semanas.

6. Un método para la biorremediación de un ambiente contaminado con s-triazinas de acuerdo a la reivindicación 4, CARACTERIZADO porque dicha s-triazina presente en el ambiente contaminado es seleccionado del

grupo que consiste de simazina, hidroxisimazina, deetilhidroxisimazina, atrazina, hidroxiatrazina, deetilatrazina, deetilhidroxiatrazina, deisopropilatrazina, fluoroatrazina, propazina, terbutilazina, ácido cianúrico y cianazina.

7. Un método de acuerdo a la reivindicación 6, CARACTERIZADA porque dicha s-triazina es una molécula que contiene un átomo de cloro.

8. Un método de acuerdo a la reivindicación 7, CARACTERIZADA porque dicha s-triazina es simazina.

9. Un método de acuerdo a la reivindicación 8, CARACTERIZADA porque dicha s-triazina es atrazina.

ANEXOS

Referencias

Hernández, M, Villalobos, P., Morgante, V. González, M. Reiff, C., E. Moore and Seeger, M. 2008. Isolation and characterization of a novel simazine-degrading bacterium from Chilean agricultural soils, *Pseudomonas* sp. MHP41. FEMs Microbiol. Lett. FEMS Microbiol Lett 286: 184-190.

(documento D3)

Morán, A. C., Muller, A., Manzano, M., González, B. 2006. Simazine treatment history determines a significant herbicide degradation potential in soils that is not improved by bioaugmentation with *Pseudomonas* sp. ADP. J. Appl. Microbiol. 101, 26-35.

(documento D4)

García-González, V., Govantes, F., Shaw, L. J., Burns, R. G., y Santero, E. 2003. Nitrogen control of atrazine utilization in *Pseudomonas* sp. strain ADP. Appl. Environ. Microbiol. 69: 6987-6993.

(documento D5)

Morgante V, López-López A, Flores C, González M, González B, Vásquez M, Rosselló-Mora R, Seeger M. Bioaugmentation with *Pseudomonas* sp. strain MHP41 promotes simazine attenuation and bacterial community changes in agricultural soils. FEMS Microbiol Ecol. 2010 Jan;71(1):114-26.

(documento D6)

RESEARCH ARTICLE

Bioaugmentation with *Pseudomonas* sp. strain MHP41 promotes simazine attenuation and bacterial community changes in agricultural soils

Verónica Morgante^{1,2}, Arantxa López-López³, Cecilia Flores^{1,2}, Myriam González^{1,2}, Bernardo González^{2,4,5}, Mónica Vásquez^{2,4}, Ramón Rosselló-Mora³ & Michael Seeger^{1,2}

¹Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Departamento de Química, Universidad Técnica Federico Santa María, Valparaíso, Chile; ²Millennium Nucleus on Microbial Ecology and Environmental Microbiology and Biotechnology EMBA, Chile; ³Marine Microbiology Group, Institut Mediterrani d'Estudis Avançats (CSIC-UIB), Esporles, Spain; ⁴Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; and ⁵Center for Advanced Studies in Ecology and Biodiversity (CASEB), Universidad Adolfo Ibáñez, Santiago, Chile

Correspondence: Michael Seeger, Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Departamento de Química, Universidad Técnica Federico Santa María, Av. España 1680, Valparaíso, Chile. Tel.: +56 32 265 4236; fax: +56 32 265 4782; e-mail: michael.seeger@usm.cl

Received 13 March 2009; revised 7 September 2009; accepted 10 September 2009.
Final version published online 2 November 2009.

DOI:10.1111/j.1574-6941.2009.00790.x

Editor: Christoph Tebbe

Keywords
bioaugmentation; simazine; *Pseudomonas* sp. MHP41; community structure; 16S rRNA gene clone libraries; FISH.

Abstract

Bioremediation is an important technology for the removal of persistent organic pollutants from the environment. Bioaugmentation with the encapsulated *Pseudomonas* sp. strain MHP41 of agricultural soils contaminated with the herbicide simazine was studied. The experiments were performed in microcosm trials using two soils: soil that had never been previously exposed to *s*-triazines (NS) and soil that had > 20 years of *s*-triazine application (AS). The efficiency of the bioremediation process was assessed by monitoring simazine removal by HPLC. The simazine-degrading microbiota was estimated using an indicator for respiration combined with most-probable-number enumeration. The soil bacterial community structures and the effect of bioaugmentation on these communities were determined using 16S rRNA gene clone libraries and FISH analysis. Bioaugmentation with MHP41 cells enhanced simazine degradation and increased the number of simazine-degrading microorganisms in the two soils. In highly contaminated NS soil, bioaugmentation with strain MHP41 was essential for simazine removal. Comparative analysis of 16S rRNA gene clone libraries from NS and AS soils revealed high bacterial diversity. Bioaugmentation with strain MHP41 promoted soil bacterial community shifts. FISH analysis revealed that bioaugmentation increased the relative abundances of two phylogenetic groups (*Acidobacteria* and *Planctomycetes*) in both soils. Although members of the Archaea were metabolically active in these soils, their relative abundance was not altered by bioaugmentation.

Introduction

Simazine [2-chloro-4,6-bis(ethylamine)-*s*-triazine] is a member of the *s*-triazine herbicides and has been used worldwide for the control of broadleaved weeds in agriculture, forestry and noncrop soils (i.e. roadsides, railways). Simazine and related *s*-triazines, such as atrazine, may persist in the environment for years, depending on climatic and edaphic factors such as temperature, soil moisture and organic carbon content, and the degradation activity of the soil native microbiota (Gunasekara *et al.*, 2007). As a consequence of its widespread application, this pesticide is frequently detected as

a pollutant in both surface and groundwater (Rousseaux *et al.*, 2003). *s*-Triazines are potential human carcinogens and endocrine disruptors (Birnbaum & Fenton, 2003; Hayes *et al.*, 2006), and recently, their use has been banned in EU member states (European Union, 2004). However, in America, these herbicides are still commonly used for the control of annual weeds in fruit plantations such as avocado, citrus and vineyards, and in forestry (Flores *et al.*, 2009).

Microorganisms play an important role in the removal of persistent organic pollutants (POPs) from the environment (Philp *et al.*, 2005). Natural microbial degradation of *s*-triazines has been reported in soils that have a history of

s-triazine herbicide application (Ostrowsky *et al.*, 1997; Yassir *et al.*, 1999; Rousseaux *et al.*, 2003; Morán *et al.*, 2006). However, the decontamination of previously unexposed soils requires the enhancement of the natural degradation (Rousseaux *et al.*, 2003). Bioaugmentation has been proposed as an effective and low-cost bioremediation technology to remove POPs, such as simazine and atrazine, and to minimize their dispersion to nonagricultural environments. In this respect, *s*-triazine-degrading bacteria have been isolated from soil, but only a few of these strains have been used for bioaugmentation (Struthers *et al.*, 1998; Newcombe & Crowley, 1999; Topp, 2001; Rousseaux *et al.*, 2003). Selection of the bacterial strain is critical for the success of bioaugmentation. A good candidate for bioaugmentation is *Pseudomonas* sp. strain MHP41, which is one of the most efficient *s*-triazine-degrading bacterial strains described (Hernández *et al.*, 2008). Bioaugmentation is often not successful (Gentry *et al.*, 2004; Thompson *et al.*, 2005), a main problem being the survival of cells added in the soil. To protect the inoculated bacteria and to improve bioremediation, encapsulation of cells has been proposed (Bouchez *et al.*, 2000; Vogel & Walter, 2002; Gentry *et al.*, 2004).

Microbial communities involved in the natural restoration of polluted environments have been analysed using culture-independent techniques (Nogales *et al.*, 1999; Paul *et al.*, 2006). Approaches such as 16S rRNA gene clone library construction and *in situ* hybridization with specific oligonucleotide probes (FISH) have proved useful for assessing the extent of soil microbial diversity and its dynamics (Nogales *et al.*, 2001; Barra-Caracciolo *et al.*, 2005). Although bioremediation of contaminated sites has been applied as an attractive, low-cost alternative to physical remediation techniques, the changes occurring in native microbial communities as a response to environmental perturbation or the impact of microorganisms introduced remain poorly known. A study of the structure of the autochthonous microbial communities and their shifts is required to understand the effects of exogenously added microorganisms on the existing microbial community composition.

In the present study, *Pseudomonas* sp. strain MHP41 was used to evaluate the bioaugmentation processes in agricultural soils contaminated with simazine. Strain MHP41 efficiently degrades simazine and is capable of growing rapidly using simazine as the sole nitrogen source, yielding a high biomass (Hernández *et al.*, 2008). This strain possesses the *s*-triazine catabolic *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes. Experiments were carried out in microcosms using two soils with different histories of *s*-triazine application, and strain MHP41 was introduced as an encapsulated biocatalyst. Simazine attenuation was followed in parallel by monitoring the bacterial community structure by 16S rRNA gene clone libraries and their changes by FISH

analysis. Archaea were also quantified by FISH in the two soils.

Materials and methods

Chemicals

Commercial simazine (Gesatop 90WG, 90% pure) was purchased from Syngenta (Greensboro, NC). Simazine (99% pure) and analytical standard simazine (> 99% pure) were purchased from Atanor S.A. (Buenos Aires, Argentina) and Dr Ehrensdoerfer-Schäfers GmbH (Augsburg, Germany), respectively.

Bacterial growth and encapsulation procedure

Pseudomonas sp. strain MHP41 was grown in minimal medium using 100 mg L⁻¹ of simazine as the sole nitrogen source (Hernández *et al.*, 2008). The minimal medium (pH 7.0) contained (L⁻¹): 10 mL of phosphate-buffered saline (PBS) stock solution (70 g Na₂HPO₄ · 2H₂O, 28 g K₂HPO₄, 5 g NaCl), 2.95 g L⁻¹ sodium succinate as the sole carbon source and 1 mL of trace element stock solution (5 g MgSO₄ · 7H₂O, 0.5 g FeSO₄ · 7H₂O, 0.25 g MnSO₄ · H₂O, 0.32 g ZnCl₂, 0.033 g CaCl₂ · 2H₂O, 0.018 g CuSO₄ · 5H₂O, 0.015 g CoCl₂ · 6H₂O, 0.325 g H₃BO₃, 0.5 g EDTA, 7.3 mL HCl 37%). Cultures were incubated at 28 °C with agitation (180 r.p.m.). Bacterial growth was monitored by measuring the turbidity at 600 nm (turbidity_{600 nm}). Viability was determined by counting CFUs on trypticase soy agar (Difco, MD). Encapsulation of strain MHP41 was performed using an alginate matrix. Cells of strain MHP41, grown until turbidity_{600 nm} ~ 0.8 (1.52 × 10⁸ CFU mL⁻¹), were harvested by centrifugation (10 000 g for 20 min at 4 °C) and washed twice with 20 mL of saline buffer solution (50 mM sodium phosphate, pH 7.0; 0.5 g L⁻¹ NaCl). Finally, the cells (turbidity_{600 nm} ~ 0.8) were resuspended in 1% w/v sterile sodium alginate solution (Loba Chemie Ltd, Mumbai, India). The mixture was added drop by drop into 400 mL of 50 mM CaCl₂ sterile solution using a 25-mL sterile syringe. Alginate beads containing cells of the strain MHP41 were hardened in the CaCl₂ solution for 30 min. Beads were recovered and washed with sterile water before addition to soil.

Soil characterization and bioaugmentation experiments in microcosms

Soil samples were collected from the surface stratum (0–20 cm depth) of two agricultural fields located in central Chile (Quillota province, Aconcagua valley). Both soils have been characterized by Flores *et al.* (2009). A soil was collected next to a flowering plant (*Dianthus caryophyllus*) plantation from an orchard without a history of *s*-triazine application (NS soil). This is a neutral (pH 7.2) and clay-loam soil, with a nitrogen content of 1.8 g kg⁻¹, a C/N

ratio of 11.5 and an organic matter (OM) content of 3.5%. A second soil was collected from an avocado (*Persea americana*) plantation that has been annually treated for > 20 years with simazine (3.5 kg simazine ha⁻¹), as a pre-emergent herbicide (AS soil). AS soil is a slightly acidic (pH 6.4) and loam soil with a nitrogen content of 2.0 g kg⁻¹ soil, a C/N ratio of 12.8 and a higher OM content (8.5%). At the time of collection, residual simazine was not detected either in NS or in AS soil. For microcosms, each soil was homogeneously treated with commercial simazine to obtain different initial concentrations of 3.5 kg simazine ha⁻¹ (~10 mg kg⁻¹, low level), according to standard farming practices, and 35 kg simazine ha⁻¹ (~100 mg kg⁻¹, high level). Each microcosm contained 150 g of soil in a 500-mL sterile flask. Microcosms were bioaugmented by inoculating encapsulated strain MHP41 (0.5 g of alginate beads containing cells of strain MHP41 were added to 150 g soil to reach a final concentration of 1×10^8 cells g⁻¹ dry soil) every 3–4 days. The soils and the alginate beads were thoroughly mixed using a sterilized spatula. In order to reduce contamination risks due to manipulation, all flasks were covered with a polyethylene foil and sampled under aseptic conditions. Microcosms were incubated at room temperature for 28 days. In control microcosms, soil was inoculated with sterile alginate beads. All treatments were performed in triplicate. Soil moisture was periodically controlled using an infrared absorption mass balance (Sartorius MH30) and maintained by sprinkling sterile water at 40–50% of the water-holding capacity. For microbial and chemical analysis, soil samples (50 g) were maintained at 4 °C. For bacterial community analysis, soil samples (10 g) were collected in sterile tubes and kept frozen at –20 °C. Statistical analysis was performed using two-way ANOVA with treatment and incubation time as factors (SYSTAT 6.1 for Windows). Differences were considered to be significant at $P \leq 0.05$.

Herbicide quantification in soil

Simazine in soil was quantified using HPLC (Agulló *et al.*, 2007; Martínez *et al.*, 2007). For simazine extraction from soil samples, 10 g of each soil sample was mixed with 20 mL of methanol solution (80% v/v, pH 2) and incubated for 2 h at room temperature. The soil particles were then separated by centrifugation (10 000 g for 1 min) and the supernatant (20 µL) was analysed for simazine quantification using a System Gold chromatograph (Beckman, Germany) equipped with a diode array detector and an RP-C18/Lichrospher 5-µm column (Supelco). Simazine was identified and quantified by comparison with an authentic standard (Flores *et al.*, 2009). The simazine removal data in soil were fitted to mathematical models using SIGMAPLOT 10.0 software (Science Software GmbH, Germany).

Enumeration of simazine-degrading microorganisms

The aerobic cultivable *s*-triazine catabolic potential of soil microbiota was determined using the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and the most-probable-number (MPN) enumeration in microtitre plates, as described previously (Dinamarca *et al.*, 2007), but with some modifications. Briefly, 1 g of soil sample was added to 9 mL of mineral medium (without simazine) and the tubes were vigorously shaken for 2 h at room temperature. Tenfold serial dilutions to extinction were prepared (in triplicate), and 100-µL aliquots of each dilution were used to inoculate the MPN microplates. *Pseudomonas* sp. strain MHP41 was used as a positive control. Cells of strain MHP41 previously killed by heating at 100 °C for 10 min were used as a negative control. The microtitre plates were incubated at 30 °C without shaking for 5 days. TTC (20 µL of a 1% w/v, TTC solution) was then added to each well. After 24 h of incubation at 30 °C, positive wells were determined based on the visualization of TTC formazan production (red colour). The number of cells per gram of dry soil was calculated using an MPN calculator program as described (Dinamarca *et al.*, 2007).

DNA extraction from soils

Community DNA was obtained from bulk soil (10 g of soil was collected in sterile tubes and kept frozen at –20 °C). DNA was extracted from 1 g of a thawed and homogenized soil sample, as described previously (Nogales *et al.*, 2001), but with some modifications. Briefly, cells were disrupted by grinding the soil sample embedded in liquid nitrogen in a ceramic mortar. Subsequently, DNA was purified using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA (concentrations of 70–90 µg µL⁻¹) was frozen at –20 °C until PCR amplification. Aliquots of purified DNA were analysed by electrophoresis on a 0.8% (w/v) agarose gel and compared with mass standards.

Amplification, cloning and sequence analysis of 16S rRNA genes

For library construction, the universal primer pair for Bacteria GM3 (5'-AGAGTTTGATCMTGGC-3') and GM4 (5'-TACCTTGTTACGACTT-3') was used. DNA was diluted (1:50) and three independent PCR reactions were carried out as reported previously (Zaballos *et al.*, 2005), decreasing the annealing temperature to 44 °C. Reconditioning reactions of 16S rRNA gene amplicons were performed as described (Thompson *et al.*, 2002). PCR products were mixed and purified using a Qiaquick PCR purification kit (Qiagen). Clone libraries were constructed using the pGEM-

T Easy Vectors System (Promega, MD) according to the manufacturer's recommendations. The plasmids of positive clones were extracted directly from a colony and amplified using the primer pair pUC/M13F and pUC/M13R provided with the kit. Finally, positive PCR products were purified (Qiaquick PCR purification kit, Qiagen) and sequenced using the primer pair GM3, 518F (5'-CCAGCAGCCGCGGTAAT-3') and GM4, according to BigDye Terminator v3.1 Cycle Sequencing Kit procedures (Applied Biosystems, CA). Ninety-nine 16S rRNA gene clones (51 from NS soil and 48 from AS soil) were partially sequenced (500–800 bp). Twenty-one almost-complete 16S rRNA gene sequences were obtained (1200–1500 bp). The sequences were submitted to the GenBank Nucleotide Sequence Database. Sequencing was performed by the 'Servicios Científico-Técnicos' of the Balearic Islands University (Mallorca, Spain) using an ABI 310 Capillary DNA Sequencer (Applied Biosystems).

Sequence analyses

Sequences were revised and corrected using SEQUENCHER 4.8 software (Gene Codes Corp., MI). The RDP CHIMERA-CHECK program (Cole *et al.*, 2003) was used to predict chimera clones. Sequences were automatically aligned and manually corrected using the ARB software package (Ludwig *et al.*, 2004). The aligned sequence dataset of the ARB-SILVA project (Pruesse *et al.*, 2007) was used as a reference. Partial sequences were inserted using the ARB parsimony tool into a previously reconstructed neighbour-joining tree of representative almost-complete 16S rRNA gene sequences. Sequences were grouped into operational taxonomic units (OTUs), assuming that one OTU includes sequences with similarity values $\geq 97\%$ (Zaballos *et al.*, 2005).

FISH analysis and quantification of total microorganisms

From each soil microcosm, 10 g of soil was fixed in $1 \times$ PBS / formaldehyde 4% for 2–6 h at 4 °C. In order to eliminate formaldehyde, the suspension was washed twice with $1 \times$ PBS by repeated centrifugation (6000 g for 5 min). The pellet was resuspended in $1 \times$ PBS/ethanol 1:1 and kept at –20 °C, until used for 4',6-diamidino-2-phenylindole (DAPI) counts and hybridization experiments. Microorganisms were detached from soil particles by sonication using a Soniprep 150 (MSE Scientific Instruments, Fisons Limited, UK) operating at 20 kHz and applying five pulses of 10 s each. Then, samples were diluted in 20 mL of $1 \times$ PBS for homogenization, and subsequently filtered throughout a 0.22- μ m pore size, 16-mm-diameter filter (Isopore GTTP, Millipore, MA). For FISH analysis, a piece of filter was cut (usually one-quarter) and hybridization with oligonucleotide probes (Table 1) was performed as described (Zárda

et al., 1997). Briefly, filters were incubated in 18 μ L of the hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulphate (SDS)] in the presence of 5.5 ng μ L⁻¹ of each probe and the probe-specific formamide concentration (Table 1) at 46 °C for 2 h. After hybridization, the filters were washed in 50 mL of buffer solution containing 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS and either 900, 450 or 80 mM NaCl depending on the formamide concentration during hybridization (0–5%, 10% and 35%, respectively), for 15 min at 48 °C, subsequently rinsed with distilled water and air-dried. Probes, competitor probes and formamide concentrations are specified in Table 1. Probe specificities were analysed using the PROBE MATCH tool of the RDP project II (<http://rdp.cme.msu.edu/>), and probe hybridization conditions were verified via the ProbeBase database (<http://www.microbial-ecology.net/probebase>). For quantification of total microbial abundances, a piece of filter was cut and stained with DAPI (1 μ g mL⁻¹) for 90 s and further washed with distilled water (5 s). Filters were finally immersed in absolute ethanol (5 s) in order to remove unspecific DAPI binding to avoid background colour. The filters were mounted onto microscope slides and a 4:1 mixture of Citifluor (Citifluor Ltd, UK) and Vecta Shield solution (Vector Laboratories, CA) was added. The preparations were stored at –20 °C until visualization. An epifluorescence microscope (Zeiss Axiophot AX10, Germany) was used for counting. Values are reported as means calculated from 15 to 20 randomly chosen microscopic fields corresponding to 600–800 DAPI-stained cells and expressed as cells g⁻¹ dry soil.

Results and discussion

To remediate POP-contaminated soils, bioaugmentation may be based on the introduction of either autochthonous or allochthonous microorganisms. These microorganisms can be used as biocatalysts for the degradation of the pollutants (Vogel, 1996; Gentry *et al.*, 2004). However, the success of a bioremediation process is restricted by various factors that often result in low efficiencies. Abiotic factors such as pH, extremes in temperature, water content and nutrient availability may influence the survival of introduced microorganisms. In addition, predation and competition by indigenous microbiota could decrease the added organisms (Bouchez *et al.*, 2000; Gentry *et al.*, 2004; Thompson *et al.*, 2005; Manzano *et al.*, 2007). In this report the effectiveness of *Pseudomonas* sp. strain MHP41 as a biocatalyst for the removal of simazine in two agricultural soils was studied. To prevent stress or predation of bacterial inocula, cells of strain MHP41 were encapsulated in alginate beads (Bouchez *et al.*, 2000; Gentry *et al.*, 2004; Vancov *et al.*, 2007). In fact, in one of these soils (AS soil), predation of added bacteria by protozoa was reported previously

Table 1. List of oligonucleotide probes and hybridization conditions used for FISH

Probe	Sequence (5'-3')*	Target RNA gene†	Specificity	Competitor	Formamide (% v/v)	Reference	Accession no.‡
EUB (I)	GCTGCGTCCCGTAGGAGT	16S	Most bacteria	No	35	Daims <i>et al.</i> (1999)	pB-00159
EUB (II)	GCAGCCACCCGTAGGTGT		Supplement to EUB 338: <i>Planctomycetales</i>				pB-00160
EUB (III)	GCTGCCACCCGTAGGTGT		Supplement to EUB 338: <i>Verrucomicrobiales</i>				pB-00161
ALF1B	CGTTCGYTCTGAGCCAG	16S	<i>Alphaproteobacteria</i> , some other <i>Proteobacteria</i> and <i>Verrucomicrobiales</i>	No	10	Manz <i>et al.</i> (1992)	pB-00017
BET42a	GCCTTCCACATTCGTTT	23S	<i>Betaproteobacteria</i>	GAM42a	35	Manz <i>et al.</i> (1992)	pB-00034
GAM42a	GCCTTCCACATTCGTTT	23S	<i>Gamma</i> proteobacteria	BET42a	35	Manz <i>et al.</i> (1992)	pB-00174
PLA886	GCCTTGCACCATCTCCC	16S	<i>Planctomycetales</i>	cPLA886	35	Neef <i>et al.</i> (1998)	pB-00284
HoAc1402	CTTTCGTGATGTGACGGG	16S	<i>Acidobacteria</i>	cHoAc1402	0-5	Juretschko <i>et al.</i> (2002)	pB-00183
PS56a	GCTGGCCTAGCCTTC	23S	<i>Pseudomonadales</i>	No	0-5	Schönduve <i>et al.</i> (1996)	pB-00289
ARCH915	GTGCTCCCCGCCAATTCCT	16S	Archaea	No	10	Stahl & Amann (1991)	pB-00027

*All probes were marked with the Cy3 fluorochrome (Thermo Electron Corporation, Germany).

†16S or 23S rRNA genes of *Escherichia coli*.

‡Accession numbers were obtained from the ProbeBase database (<http://www.microbial-ecology.net/probebase/>).

(Manzano *et al.*, 2007). Bioaugmentation was studied in agricultural soils with different physical properties and a history of herbicide application. NS soil is a clay-loam soil with a 3.5% OM content and that not have been treated with s-triazine herbicides, whereas AS soil is a loam soil with an 8.5% OM content and a long history of s-triazine application. Clay minerals and OM are involved in the retention of pesticides in soil, influencing their bioavailability and biological degradation (Florès *et al.*, 2009). In both soils, sorption of simazine was moderate and reversible (Flores *et al.*, 2009), and therefore simazine was available for microbial degradation.

Effect of bioaugmentation with strain MHP41 on simazine removal in soils

In a first approach, bioaugmentation trials were carried out in microcosms using NS and AS soils spiked with $\sim 10 \text{ mg kg}^{-1}$ simazine, according to the standard farming practices for weed control. Figure 1a shows the dynamics of simazine attenuation in NS soil contaminated with $\sim 10 \text{ mg kg}^{-1}$ simazine and the improvement in removal due to bioaugmentation. In the control treatment, the simazine concentration decreased $\sim 55\%$ within 28 days at a degradation rate of $0.20 \text{ mg kg}^{-1} \text{ day}^{-1}$. Bioaugmentation with *Pseudomonas* sp. MHP41 increased simazine removal to 89%. Addition of strain MHP41 significantly reduced the

half-life of simazine and increased the degradation rate twofold ($0.40 \text{ mg kg}^{-1} \text{ day}^{-1}$) ($P \leq 0.05$). The numbers of aerobic cultivable simazine-degrading microorganisms in these soil microcosms are shown in Fig. 1b. Application of a low level of simazine ($\sim 10 \text{ mg kg}^{-1}$) did not increase the number of cultivable simazine-degrading cells in NS soil during the first 3 weeks of the experiment. Only after 4 weeks were cultivable simazine-degrading cells detected. Of note, bioaugmentation with encapsulated strain MHP41 significantly increased ($P \leq 0.05$) the number of cultivable simazine-degrading microbial cells ($4.5 \times 10^2 \text{ cells g}^{-1}$). In agreement, a low number of indigenous atrazine-degrading microorganisms was reported by Struthers *et al.* (1998) in soils nonadapted to atrazine, but these microorganisms increased after inoculation of a biocatalyst into the soil.

Figure 2a shows simazine removal and the dynamics of the cultivable simazine-degrading microorganisms in the AS soil during bioaugmentation experiments. Simazine attenuation was observed in this soil (degradation rate of $0.45 \text{ mg kg}^{-1} \text{ day}^{-1}$). However, bioaugmentation reduced the simazine half-life from 11 to 3 days and increased the simazine removal rate to $0.80 \text{ mg kg}^{-1} \text{ day}^{-1}$ ($P \leq 0.05$). Simazine application increased the cultivable simazine-degrading microorganisms in the AS soil from undetectable (day 0) to $2.3 \times 10^3 \text{ cells g}^{-1}$ after 23 days (Fig. 2b). This increase of simazine-degrading microorganisms was much higher than that observed in NS soil. This might be an

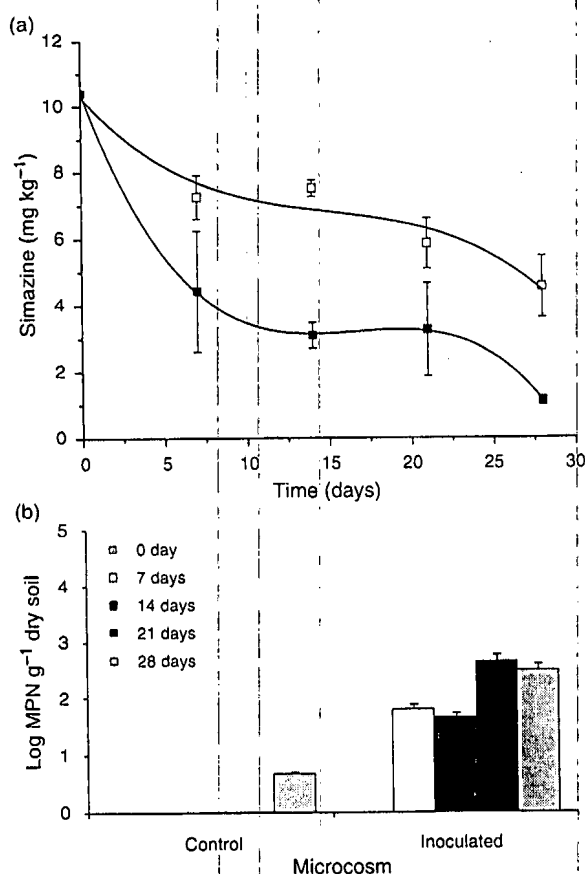


Fig. 1. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in a soil without a history of *s*-triazine application (NS). The soil was spiked with $\sim 10 \text{ mg kg}^{-1}$ simazine. (a) Simazine removal as determined by HPLC. Control treatments ($\square$) were performed using sterile alginate beads. Inoculation treatments ($\blacksquare$) were performed with encapsulated cells of the strain MHP41. (b) Simazine-degrading microbiota as estimated by MPN. Each point represents the average of three independent experiments. Vertical bars indicate SD.

indication that the autochthonous-degrading microbiota thrives in detectable numbers in the soil with a previous treatment history, but probably remains dormant. These results are in agreement with previous reports indicating that the *s*-triazine treatment history of soils determines the intrinsic *s*-triazine-degrading potential (Yassir *et al.*, 1999; Rhine *et al.*, 2003; Morán *et al.*, 2006). Bioaugmentation with encapsulated strain MHP41 in the AS soil showed a significant increase ($P \leq 0.05$) in the cultivable simazine-degrading microorganisms, reaching values of $7.1 \times 10^3 \text{ cells g}^{-1}$ at the final stages of the experiment.

In agreement with our results, previous reports have demonstrated that bioaugmentation with *Pseudaminobacter* sp. strain C147, *Nocardioide* sp. C190 and *Chelatobacter heintzii* Cit1 increased atrazine removal in soils without a history of herbicide treatment (Topp, 2001; Rousseaux *et al.*,

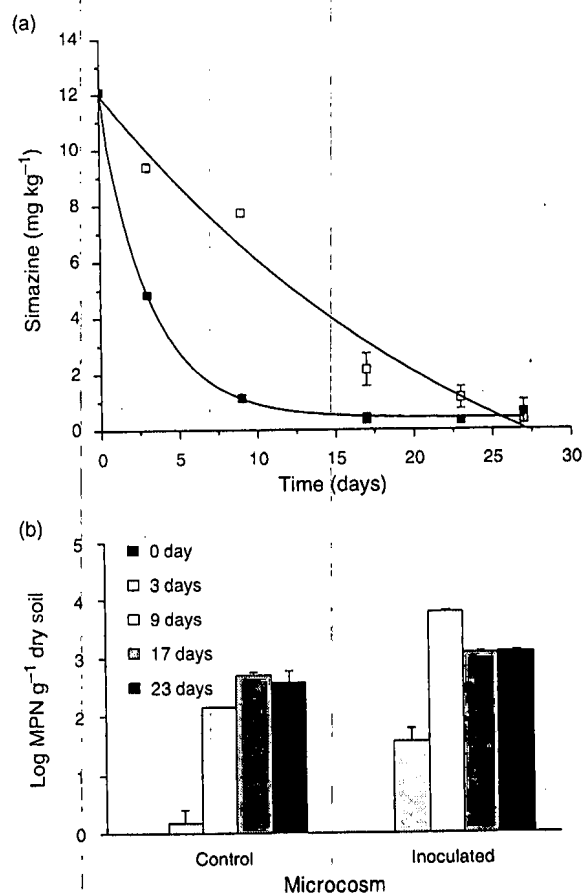


Fig. 2. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in a soil with a history of simazine application (AS). The soil was spiked with $\sim 10 \text{ mg kg}^{-1}$ simazine. (a) Simazine removal as determined by HPLC. Control treatments ($\square$) were performed using sterile alginate beads. Inoculation treatments ($\blacksquare$) were performed with encapsulated cells of the strain MHP41. (b) Simazine-degrading microbiota as estimated by MPN. Each point is an average of three independent experiments. Vertical bars indicate SD.

2003). In addition, *Agrobacterium radiobacter* J14a accelerated the atrazine degradation rate in soils without and with previous herbicide application (Struthers *et al.*, 1998). Conversely, *Pseudomonas* sp. strain ADP was unable to increase atrazine or simazine degradation in *s*-triazine-polluted soil (Topp, 2001; Morán *et al.*, 2006). NS and AS soils have a nitrogen content of 1.8 and 2.0 g kg^{-1} soil, respectively. Although nitrogen sources in soils can influence simazine degradation by bacteria (García-González *et al.*, 2003; Barra-Caracciolo *et al.*, 2005), in this study, simazine removal by strain MHP41 was found in both soils.

Despite the fact that the two agricultural soils harbour autochthonous microorganisms with simazine catabolic potential, simazine removal was significantly increased after bioaugmentation with strain MHP41. Allochthonous

microorganisms that are reintroduced in soils show unsatisfactory bioremediation potential (Atlas & Unterman, 1999; Rousseaux *et al.*, 2003). However, strain MHP41 is an autochthonous bacterium (Hernández *et al.*, 2008). In this study, the use of an autochthonous bacterial strain, together with the encapsulation of cells in alginate (Vogel & Walter, 2002; Vancov *et al.*, 2005), could explain the enhanced simazine removal rates in inoculated soils. In contrast, it has been reported that bioaugmentation with free cells of the allochthonous bacterium *Pseudomonas* sp. strain ADP in AS soil was not successful (Morán *et al.*, 2006).

Effect of bioaugmentation with strain MHP41 on simazine attenuation in soil contaminated with a high concentration of simazine

As the results showed that bioaugmentation successfully removed $\sim 10 \text{ mg kg}^{-1}$ simazine in the two soils, investigation was further focused on the effectiveness of bioaugmentation with strain MHP41, using the nonperturbed NS soil, experimentally contaminated with a 10-fold higher simazine concentration. In this case, natural attenuation of simazine was not observed in noninoculated treatments (Fig. 3a). This is in agreement with the low number of simazine-degrading microorganisms detected after 21 days (Fig. 3b). Remarkably, bioremediation with the encapsulated strain MHP41 was successful in achieving complete simazine removal (99%) after 28 days, with a high degradation rate of $4.15 \text{ mg kg}^{-1} \text{ day}^{-1}$ (Fig. 3a). It has been reported that in highly contaminated soils, *A. radiobacter* strain J14a was able to remove almost 50% of the 200 mg kg^{-1} atrazine, whereas in control soils the natural attenuation was lower (29%). By contrast, in soils contaminated with $\sim 160\text{--}300 \text{ mg kg}^{-1}$ of atrazine, removal of the herbicide by *Pseudomonas* sp. strain ADP ($2 \times 10^7 \text{ cells g}^{-1}$) was limited (Struthers *et al.*, 1998). The present results indicate that bioaugmentation with encapsulated strain MHP41 was successful with a 10-fold higher concentration of contaminant in a soil that had an intrinsic, but low potential of simazine attenuation. Our results reinforce the use of bioaugmentation as an appropriate bioremediation strategy, especially when natural attenuation in the soil is low or undetectable.

Assessment of the bacterial community diversity and structure in agricultural soils

The bacterial communities of the two original sampled agricultural soils were characterized through 16S rRNA gene clone library analyses before the microcosm experiments. Two 16S rRNA gene clone libraries were constructed with NS and AS soil DNA. Ninety-nine 16S rRNA gene clones (51

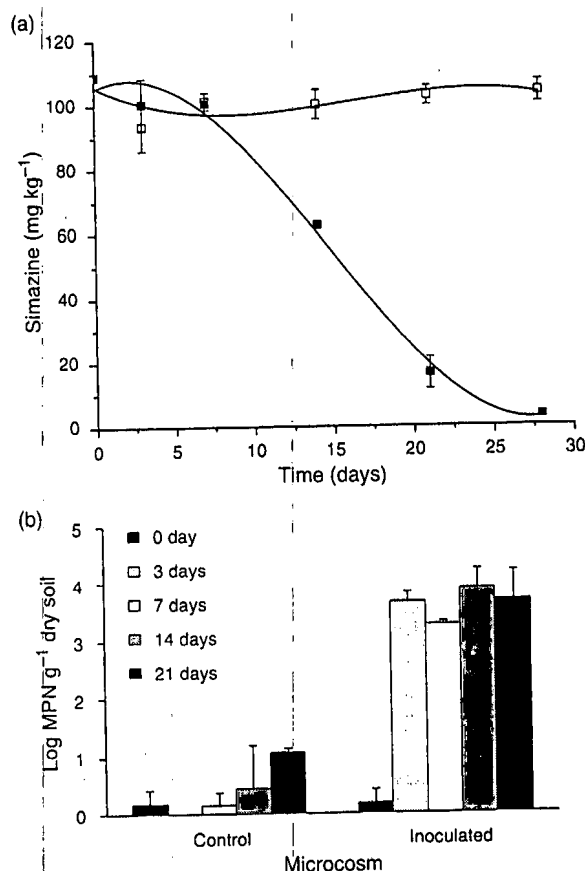


Fig. 3. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in NS soil contaminated with a high concentration of simazine. The soil was spiked with $\sim 100 \text{ mg kg}^{-1}$ simazine. (a) Simazine removal as determined by HPLC. Control treatments ($\square$) were performed using sterile alginate beads. Inoculation treatments ($\blacksquare$) were performed with encapsulated cells of the strain MHP41 in alginate. (b) The simazine-degrading microbiota as estimated by MPN. Each point is an average of three independent experiments. Vertical bars indicate SD.

from NS soil and 48 from AS soil) were partially sequenced (500–800 bp) (Table 2). The affiliations of clone sequences with those of the described taxa indicated the presence of mainly aerobic organisms. The degree of similarity of these sequences to their closest relatives in the database was in the range 80–99%. Sequence similarity to cultivated species was between 80% and 90% in most cases, whereas similarity to uncultured clones was often close to 99%. In addition, 21 clones of representative phylogenetic groups from the two clone libraries were fully sequenced (Table 2). Bacterial diversity coverage achieved by clone sequencing was evaluated using rarefaction analyses, assuming that one OTU is formed by the sequences that present a similarity $\geq 97\%$ (Zaballos *et al.*, 2005). The analysis indicated high bacterial sequence diversity, as no asymptotic curves could be observed. Additional sequencing would be necessary to achieve

Table 2. List of partial and nearly complete sequenced phylotypes obtained through clone library studies on 16S rRNA genes

Clone cluster (%)	Partial clones accession no.*	Percentage per soil type†	Nearly complete clone ID	Accession no.*	Percentage identity	Best match or closest related type strain	Database accession no.‡
Acidobacteria	FJ561545–FJ561554	19.5 ^{NS}	C003	FJ468375	94.0	<i>Acidobacteria bacterium</i> Ellin6099	AY234751
			C064	FJ468380	89.2	<i>Acidobacteria bacterium</i> Ellin7246	AM749787
	FJ561497–FJ561510	29.5 ^{AS}	P011	FJ468386	87.5	<i>Acidobacteriaceae</i> <i>bacterium</i> K22	AM749787
			P028	FJ468392	83.2	<i>Holophaga foetida</i> strain TMBS4 ^T (DSM 6591 ^T)	X77215
Actinobacteria	FJ561555–FJ561560	12 ^{NS}					
	FJ561511–FJ561513	6.5 ^{AS}	P021	FJ468388	70.8	<i>Georgenia muralis</i>	EF154258
Alphaproteobacteria	FJ561561–FJ561572	23 ^{NS}	C063	FJ468379	90.5	<i>Stella vacuolata</i> , strain DSM 5901	AJ535711
			C078	FJ468382	93.0	<i>Mesorhizobium</i> <i>plurifarum</i> strain ORS10056	Y14161
Betaproteobacteria	FJ561514–FJ561522	19 ^{AS}					
	FJ561573–FJ561577	10 ^{NS}					
	FJ561523–FJ561527	10 ^{AS}	P006	FJ468384	91.9	<i>Burkholderia</i> sp. strain NK8	AB208548
Gammaproteobacteria	FJ561578–FJ561579	4 ^{NS}					
	FJ561528–FJ561531	8.5 ^{AS}	P026	FJ468390	88.2	<i>Legionella pneumophila</i>	CR628337
			P046	FJ468395	93.3	<i>Xanthomonas campestris</i> strain ATCC 33913	AE012505
Deltaproteobacteria	FJ561580	2 ^{NS}					
	FJ561532–FJ561534	6.5 ^{AS}	P007	FJ468385	81.2	<i>Syntrophus</i> <i>aciditrophicus</i> SB	CP000252
			P012	FJ468387	85.6	<i>Geobacter</i> <i>sulfurreducens</i> PCA	AE017180
Bacteroidetes	FJ561581–FJ561583	6 ^{NS}	P024	FJ468389	88.6	<i>Haliangium tepidum</i>	AB062751
			C022	FJ468377	81.8	<i>Thermonema rossianum</i> strain NR-27T (DSM 10300)	Y08956
Chloroflexi	FJ561535	2 ^{AS}					
	FJ561584–FJ561586	6 ^{NS}	C053	FJ468378	82.0	<i>Dehalococcoides</i> sp. strain BH180-15	AJ431246
Firmicutes	FJ561587–FJ561592	12 ^{NS}	C007	FJ468376	81.3	<i>Desulfosporosinus</i> <i>orientis</i>	AJ493052
Nitrospirae	FJ561593–FJ561594	4 ^{NS}					
	FJ561536	2 ^{AS}	P045	FJ468394	95.3	<i>Nitrospira</i> sp.	AJ224039
Planctomycetes	FJ561595	2 ^{NS}	C074	FJ468381	94.2	<i>Planctomycetes</i> sp.	AY673410
	FJ561537–FJ561541	10 ^{AS}	P027	FJ468391	87.5	<i>Planctomyces maris</i>	X62910
			P029	FJ468393	87.3	<i>Pirellula</i> sp.	X81947
Verrucomicrobia	FJ561543	2 ^{AS}	P001	FJ468383	84.6	<i>Prostheco bacter</i> <i>debontii</i> (DSM 14044)	AJ966882
Gemmatimonadetes	FJ561542	2 ^{AS}					
Unknown Bacteria	FJ561544	2 ^{AS}					
Total clones	99		21				

The list provides the accession numbers of the representative clones from both soils and the closest match or type strain available in the SILVA database (<http://www.arb-silva.de>). Clones retrieved from agricultural soil with different history of simazine application are indicated. Accession numbers of the partial and nearly complete sequences have been listed along with accession numbers of the closest best match.

*GenBank database.

†NS, soil not previously exposed to s-triazines. AS, soil with a long history of s-triazine application.

‡SILVA-SSU database accession number.

better coverage of the diversity of the amplified 16S rRNA genes. However, it was assumed that in this case, the most abundant phylotypes thriving in the agricultural soils were identified.

Bacterial diversity and community structure changes in NS soil due to bioaugmentation

The bacterial diversity in NS soil is shown in Fig. 4a. The NS clone library was dominated by members of the phylum *Proteobacteria* (39%), most of them affiliated with the *Alphaproteobacteria* and *Betaproteobacteria*. Twenty-three per cent of the clones were affiliated with the *Alphaproteobacteria*, most of them (60%) relatives associated with the family *Rhizobiales*. The *betaproteobacterial* OTUs detected were dominated by members of the order *Burkholderiales*, while *Gammaproteobacteria* and *Deltaproteobacteria* were much less well represented. *Acidobacteria* comprised 19% of the library, with most of the sequences affiliating with uncultured strains (> 96% identity). Other represented groups were *Actinobacteria* (12%), *Firmicutes* (12%), *Chloro-*

flexi (6%), *Bacteroidetes* (6%), *Nitrospirae* (4%) and *Planctomycetes* (2%).

Bacterial community structure dynamics during the bioaugmentation with encapsulated strain MHP41 was studied using FISH analysis. The selection of the phylogenetic probes for FISH was based on the major representative groups found in the clone libraries. In addition, an Archaea-specific probe (Arch915) was used. The total bacterial abundances estimated by DAPI counts were relatively high in NS soil, ranging from 1.5 to 2.1×10^{10} cells g⁻¹ dry soil. At time 0 of the bioremediation experiment in NS soil, 50% of the cell abundance was assigned to the Bacteria domain (EUB338 probe mixture; Fig. 4b). This value increased by a factor of 1.5 in the bioaugmented soil after 28 days of inoculation. In the control soil, a similar increase (factor of 1.3) in the bacterial fraction was observed. Archaea (Arch915-stained cells) accounted for only 5% of the total abundance ($6-7 \times 10^8$ cells g⁻¹) in control and inoculated soils, and no changes were observed due to the bioaugmentation. Bacteria hybridizing with probes Alf1b (targeting *Alphaproteobacteria*), HoAc1402 (targeting *Acidobacteria*) and Pla886 (targeting *Planctomycetes*) were detected in large numbers in NS soils. At time 0, the *Alphaproteobacteria* dominated (10–20%), followed by *Planctomycetes* (about 11%), *Acidobacteria* (9%), *Betaproteobacteria* (9%) and *Gammaproteobacteria* (1–2%) (Fig. 4b). In these soils, *Pseudomonas* populations (tested with probe PS56a) were detected sporadically and only in numbers that remained around the detection limit (set at 1% of the DAPI-stained cells). No increase was observed after either bioaugmentation or simazine application treatment.

The addition of simazine only produced a clear increase in the *alphaproteobacterial* fraction (about 2.5-fold). Bioaugmentation with strain MHP41 promoted changes in some of the most abundant groups detected by FISH (Fig. 4b), most notably the *Planctomycetes* and *Acidobacteria* (increasing almost threefold after 28 days of incubation), followed by *Gammaproteobacteria* (twofold).

These results suggest that *Acidobacteria* and *Planctomycetes* could be active simazine-degrading bacteria or may play an important role in the functionality of the bacterial community in these soils. Despite their abundance in the biosphere, knowledge of the *Planctomycetes* and *Acidobacteria* is limited by the fact that the majority of these microorganisms are uncultured (Quaiser et al., 2003; Buckley et al., 2006). To understand the role of *Planctomycetes* and *Acidobacteria* in agricultural soils, a novel insight into the phenotypic or the physiological characterization of these bacterial groups is desirable.

In the present study, a significant increase in *pseudomonads* after repeated introduction of 10^8 cells g⁻¹

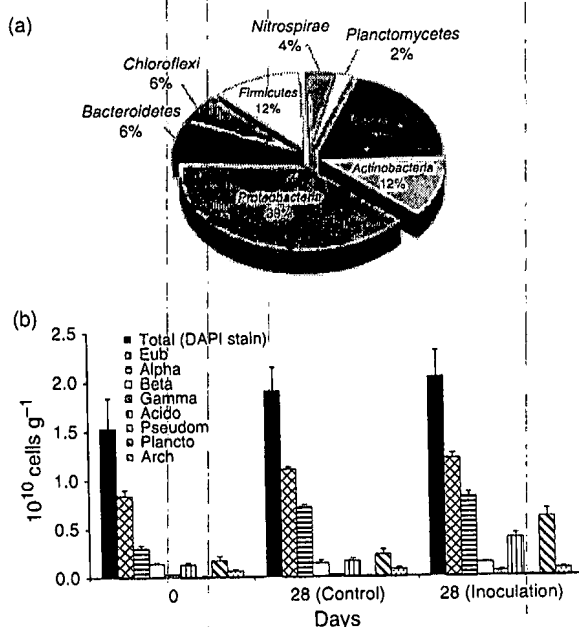


Fig. 4. Bacterial community diversity and dynamics during bioaugmentation in NS soil. (a) Representative phylogenetic groups retrieved from NS soil by the clone library. Values are shown as per cent of the total clones in each clone library. (b) FISH analysis of the microbial community from the NS soil amended with simazine (~ 100 mg kg⁻¹) and bioaugmented with *Pseudomonas* sp. strain MHP41. Each value is an average of three independent experiments. Vertical bars indicate SD. Total (DAPI stain), total microbial abundances; Eub, Bacteria; Alpha, *Alphaproteobacteria*; Beta, *Betaproteobacteria*; Gamma, *Gammaproteobacteria*; Acido, *Acidobacteria*; Pseudom, *Pseudomonas*; Plancto, *Planctomycetes*; Arch, Archaea.

of MHP41 in the soils was not observable. This is not surprising as pseudomonads do not generally account for a significant part of soil communities (Nogales *et al.*, 1999; Lloyd-Jones *et al.*, 2005; Martin-Laurent *et al.*, 2006). However, *Pseudomonas* strains have commonly been isolated from such environments (Cho & Tiedje, 2000; Lloyd-Jones *et al.*, 2005).

Bacterial diversity and community structure changes in AS soil due to bioaugmentation

In AS soil, bacterial diversity was dominated by members of the phylum *Proteobacteria*, most of them affiliated with *Alphaproteobacteria* and *Betaproteobacteria* (around 29%) as described previously for NS soil. Thus, *Gammaproteobacteria* and *Deltaproteobacteria* were not abundant. *Acidobacteria* and *Actinobacteria* were represented in the AS clone library, comprising 29% and 7% of the clones, respectively. Members of the phylum *Planctomycetes* were much more abundant in AS soil than in NS soil (10% vs. 2%). Some other minor phylogenetic groups such as *Bacteroidetes*, *Nitrospirae*, *Verrucomicrobia* and *Gemmatimonadetes* were observed. *Firmicutes* and *Chloroflexi* were not detected in soil AS (Fig. 5a). The total bacterial abundance in AS soil (DAPI counts) ranged from 2.1 to 3.0×10^{10} cells g^{-1} dry soil, and only 40% was assigned to the Bacteria domain at time 0 (Fig. 5b). After 28 days, Bacteria cells counts (EUB338 probe mixture) increased in bioaugmented soil by a factor of 1.8 compared with 1.3 in the control soil. The AS soil, which has a long history of simazine treatment, experienced similar changes in the bacterial community by bioaugmentation as the NS soil (Fig. 5b). After 28 days, inoculations of the strain MHP41 increased the relative abundances of *Alphaproteobacteria* (almost threefold), *Planctomycetes* (almost 2.5-fold), and *Acidobacteria* (almost 1.5-fold) whereas simazine application promoted only a slight increase in *Alphaproteobacteria* and *Planctomycetes* cells after 28 days (both < 1.5-fold). The relative abundance of Archaea did not change as a result of bioaugmentation (remaining at ~5% of the total).

The results indicated that despite the fact that pseudomonads (and, in this case, the inoculated *Pseudomonas* sp. strain MHP41) are not present in significant numbers in these soils, bioaugmentation with strain MHP41 promoted bacterial community shifts in both AS and NS soils. These changes at the bacterial community level could not be linked to the intrinsic agricultural soil composition or to the previous history of simazine application, but rather to the introduction of the simazine-degrading strain MHP41. To our knowledge, this is the first report showing bacterial community structure changes due to bioaugmentation in simazine-contaminated soil. In a previous report, FISH was

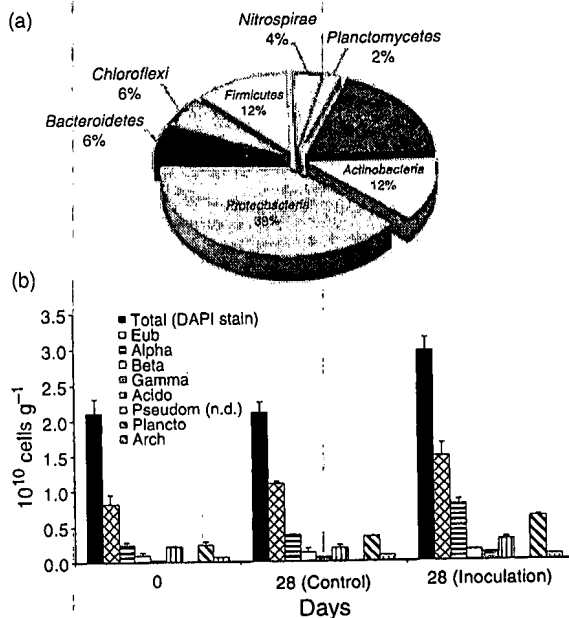


Fig. 5. Bacterial community diversity and dynamics during bioaugmentation in AS soil. (a) Representative phylogenetic groups retrieved from AS soil by the clone library. Values are shown as per cent of total clones in each clone library. (b) FISH analysis of the microbial community from AS soil spiked with simazine (~ 10 mg kg^{-1}) and bioaugmented with *Pseudomonas* sp. strain MHP41. Each value is an average of three independent experiments. Vertical bars indicate SD. Total (DAPI stain), total microbial abundances; Eub, Bacteria; Alpha, *Alphaproteobacteria*; Beta, *Betaproteobacteria*; Gamma, *Gammaproteobacteria*; Acido, *Acidobacteria*; Pseudom, *Pseudomonas*; Plancto, *Planctomycetes*; Arch, Archaea.

used to describe shifts in the existing community structure caused by the addition of the herbicide simazine and urea to soil (Barra-Caracciolo *et al.*, 2005). In this case, the groups most influenced were the *Alphaproteobacteria* and *Betaproteobacteria*. The fact that in our study the major changes occurred after bioaugmentation, rather than by simazine application, may indicate that the response is mainly due to the inoculated strain. In this respect, the bioavailability of nitrogen will probably be enhanced after simazine degradation into more soluble nitrogen-containing products (Cook & Hütter, 1981; Mandelbaum *et al.*, 1995; Rhine *et al.*, 2003) and may thus directly influence the community structure changes.

Conclusions

Bioremediation using *Pseudomonas* sp. strain MHP41 was successful for the removal of simazine at the scale of microcosms. Removal of simazine by bioaugmentation was independent of the previous history of soil contamination. Of note, strain MHP41 was essential for simazine removal in highly contaminated NS soil. The autochthonous bacterial

communities in the soil were also clearly influenced as a result of bioaugmentation. *Planctomycetes* and *Acidobacteria* showed detectable increased numbers via FISH as a common response to the treatment, independent of the quality and the history of the soil. However, the presence of the introduced strain could not be detected in the soils, and their numbers remained at the limits of FISH detection. The presence of archaeal lineages as metabolically active members of microbial communities in the two soils indicates the ecological significance of Archaea in agricultural soils. Together, the results indicate that bioaugmentation with strain MHP41 significantly and effectively increased the rates of bioremediation over the native autochthonous catabolic activity, and the soil bacterial community changes may respond to catalysis of the contaminant rather than to distortion due to addition of cell biomass.

Acknowledgements

We thank Pola Miralles for analysis and discussion and Marco Mendez for statistical analysis advice. We gratefully acknowledge financial support from the Milenio P04/007-F (ICM, MIDEPLAN-Chile), USM 130522, USM 130836, USM 130948, CIT Nanotechnology and Systems Biology, PBCT Red 12 and INCO ICA4-CT-2002-10011 (European Union) grants. V.M. gratefully acknowledges MECESUP-UCV-0206 and Milenio EMBA fellowships. R.R.-M. and A.L.-L. acknowledge the financial support of the projects CE-CSD2007-0005 (Consolider Ingenio 2010) and CLG2006-12714-G02-02 of the Spanish Ministry of Science and Innovation, and the exchange project 2006CL0032 between the Spanish Council (CSIC) and the University of Chile.

References

- Agulló L, Cámara B, Martínez P, Latorre-Reyes V & Seeger M (2007) Response to (chloro)biphenyls of the PCB-degrader *Burkholderia xenovorans* LB400 involves stress proteins induced also by heat shock and oxidative stress. *FEMS Microbiol Lett* 267: 167–175.
- Atlas RM & Unterman R (1999) Bioremediation. *Manual of Industrial Microbiology and Biotechnology*, 2nd edn (Demain AL & Davies JE, eds), pp. 666–681. ASM Press, Washington, DC.
- Barra-Caracciolo AB, Grenni P, Ciccoli R, Di Landa G & Cremisini C (2005) Simazine biodegradation in soil: analysis of bacterial community structure by *in situ* hybridization. *Pest Manag Sci* 61: 863–869.
- Birnbaum LS & Fenton SE (2003) Cancer and developmental exposure to endocrine disruptors. *Environ Health Persp* 111: 389–394.
- Bouchez T, Patureau D, Dabert P et al. (2000) Ecological study of a bioaugmentation failure. *Environ Microbiol* 2: 179–190.
- Buckley DH, Huangyutitham V, Nelson TA, Rumberger A & Thies JE (2006) Diversity of *Planctomycetes* in soil in relation to soil history and environmental heterogeneity. *Appl Environ Microb* 72: 4522–4531.
- Cho JC & Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. *Appl Environ Microb* 66: 5448–5456.
- Cole JR, Chai B, Marsh TL et al. (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* 31: 442–443.
- Cook AM & Hütter R (1981) s-Triazines as nitrogen source for bacteria. *J Agr Food Chem* 29: 1135–1143.
- Daims H, Bruhl A, Amann R, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 22: 434–444.
- Dinamarca MA, Cereceda-Balic F, Fadic X & Seeger M (2007) Analysis of s-triazine-degrading microbial communities in soils using most-probable-number enumeration and tetrazolium-salt detection. *Int Microbiol* 10: 209–215.
- European Union (2004) Commission Decision of 10 March 2004 concerning the non-inclusion of simazine in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing this active substance. *Official Journal of the European Union L78* 16 March 2004, pp. 50–52.
- Flores C, Morgante V, González M, Navia R & Seeger M (2009) Adsorption studies of the herbicide simazine in agricultural soils of the Aconcagua valley, central Chile. *Chemosphere* 74: 1544–1549.
- García-González V, Govantes F, Shaw LJ, Burns RG & Santero E (2003) Nitrogen control of atrazine utilization in *Pseudomonas* sp. strain ADP. *Appl Environ Microb* 69: 6987–6993.
- Gentry TJ, Rensing C & Pepper IL (2004) New approaches for bioaugmentation as a remediation technology. *Crit Rev Env Sci Tec* 34: 447–494.
- Gunasekara AS, Troiano J, Goh KS & Tjeerdema RS (2007) Chemistry and fate of simazine. *Rev Environ Contam T* 189: 1–23.
- Hayes TB, Stuart AA, Mendoza M et al. (2006) Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17beta-estradiol): support for the demasculinization/feminization hypothesis. *Environ Health Persp* 114 (suppl 1): 134–141.
- Hernández M, Villalobos P, Morgante V, González M, Reiff C, Moore E & Seeger M (2008) Isolation and characterization of a novel simazine-degrading bacterium from agricultural soil of central Chile, *Pseudomonas* sp. MHP41. *FEMS Microbiol Lett* 286: 184–190.
- Juretschko S, Loy A, Lehner A & Wagner M (2002) The microbial community composition of a nitrifying–denitrifying activated sludge from an industrial sewage treatment plant

- analyzed by the full-cycle rRNA approach. *Syst Appl Microbiol* 25: 84–99.
- Lloyd-Jones G, Laurie AD & Tizzard AC (2005) Quantification of the *Pseudomonas* population in New Zealand soils by fluorogenic PCR assay and culturing techniques. *J Microbiol Meth* 60: 217–224.
- Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32: 1363–1371.
- Mandelbaum RT, Allan DL & Wackett LP (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the triazine herbicide atrazine. *Appl Environ Microb* 61: 1451–1457.
- Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligonucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* 15: 593–600.
- Manzano M, Morán AC, Tesser B & González B (2007) Role of eukaryotic microbiota in soil survival and catabolic performance of the 2,4-b herbicide degrading bacteria *Cupriavidus necator* JMP134. *Antonie van Leeuwenhoek* 91: 115–126.
- Martínez P, Agulló L, Hernández M & Seeger M (2007) Chlorobenzoate inhibits growth and induces stress proteins in the PCB-degrading bacterium *Burkholderia xenovorans* LB400. *Arch Microbiol* 188: 289–297.
- Martin-Laurent F, Barrés B, Wagschal I, Piutti S, Devers M, Soulas G & Philippot L (2006) Impact of the maize rhizosphere on the genetic structure, the diversity and the atrazine-degrading gene composition of cultivable atrazine-degrading communities. *Plant Soil* 282: 99–115.
- Morán AC, Muller A, Manzano M & González B (2006) Simazine treatment history determines a significant herbicide degradation potential in soils that is not improved by bioaugmentation with *Pseudomonas* sp. ADP. *J Appl Microbiol* 101: 26–35.
- Neef A, Amann R, Schlesner H & Schleifer KH (1998) Monitoring a widespread bacterial group: *in situ* detection of *Planctomycetes* with 16S rRNA-targeted probes. *Microbiology* 144: 3257–3266.
- Newcombe DA & Crowley DE (1999) Bioremediation of atrazine-contaminated soil by repeated applications of atrazine-degrading bacteria. *Appl Environ Microb* 51: 877–882.
- Nogales B, Moore ER, Abraham WR & Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environ Microbiol* 1: 199–212.
- Nogales B, Moore ER, Llobet-Brossa E, Rossello-Mora R, Amann R & Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microb* 67: 1874–1884.
- Ostrofsky EB, Traina SJ & Tuovinen OH (1997) Variation in atrazine mineralization rates in relation to agricultural management practice. *J Environ Qual* 26: 647–657.
- Paul D, Pandey G, Meier C, van der Meer JR & Jain RK (2006) Bacterial community structure of a pesticide-contaminated site and assessment of changes induced in community structure during bioremediation. *FEMS Microbiol Ecol* 57: 116–127.
- Philp JC, Bamforth SM, Singleton I & Atlas RM (2005) Environmental pollution and restoration: a role for bioremediation. *Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup* (Atlas RM & Philp JC, eds), pp. 1–48. ASM Press, Washington, DC.
- Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 35: 7188–7196.
- Quaiser A, Ochsenreiter T, Lanz C, Schuster SC, Treusch AH, Eck J & Schleper C (2003) *Acidobacteria* form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Mol Microbiol* 50: 563–575.
- Rhine ED, Fuhrmann JJ & Radosevich M (2003) Microbial community responses to atrazine exposure and nutrient availability: linking degradation capacity to community structure. *Microb Ecol* 46: 145–160.
- Rousseaux S, Hartmann A, Lagacherie B, Piutti S, Andreux F & Soulas G (2003) Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1, in four different soils: effects of different inoculum densities. *Chemosphere* 51: 569–576.
- Schöndube P, Sara M & Friedl A (1996) Influence of physiologically relevant parameters on biomass formation in a trickle-bed bioreactor used for waste gas cleaning. *Appl Microbiol Biot* 45: 286–292.
- Stahl DA & Amann R (1991) Development and application of nucleic acid probes. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205–248. John Wiley & Sons Ltd, Chichester, UK.
- Struthers JK, Jayachandran K & Moorman TB (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *Appl Environ Microb* 64: 3368–3375.
- Thompson IP, van der Gast CJ, Ciric L & Singer AC (2005) Bioaugmentation for bioremediation: the challenge of strain selection. *Environ Microbiol* 7: 909–915.
- Thompson JR, Marcelino LA & Polz MF (2002) Heteroduplexes in mixed-templated amplifications: formation, consequence and elimination by reconditioning PCR. *Nucleic Acids Res* 30: 2083–2088.
- Topp E (2001) A comparison of three atrazine-degrading bacteria for soil bioremediation. *Biol Fert Soils* 33: 529–534.
- Vancov T, Jury K & Van Zwieten L (2005) Atrazine degradation by encapsulated *Rhodococcus erythropolis* NI86/21. *J Appl Microbiol* 99: 767–775.
- Vancov T, Jury K, Rice N, Van Zwieten L & Morris S (2007) Enhancing cell survival of atrazine degrading *Rhodococcus erythropolis* NI86/21 cells encapsulated in alginate beads. *J Appl Microbiol* 102: 212–220.

- Vogel TM (1996) Bioaugmentation as a soil bioremediation approach. *Curr Opin Biotech* 7: 311–316.
- Vogel TM & Walter MV (2002) Bioaugmentation. *Manual of Environmental Microbiology* (Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ & Stetzenbach LD, eds), pp. 952–971. ASM Press, Washington, DC.
- Yassir A, Lagacherie B, Houot S & Soulas G (1999) Microbial aspects of atrazine biodegradation in relation to history of soil treatment. *Pestic Sci* 55: 799–809.
- Zaballos M, López-López A, Ovreas L *et al.* (2005) Comparison of prokaryotic diversity at offshore oceanic locations reveals a different microbiota in the Mediterranean Sea. *FEMS Microbiol Ecol* 56: 389–405.
- Zarda B, Hahn D, Chatzinotas A, Schönhuber W, Neef A, Amann R & Zeyer J (1997) Analysis of bacterial community structure in bulk soil by *in situ* hybridization. *Arch Microbiol* 168: 185–192.

Nitrogen Control of Atrazine Utilization in *Pseudomonas* sp. Strain ADP

Vicente García-González,¹ Fernando Govantes,^{1*} Liz J. Shaw,² Richard G. Burns,² and Eduardo Santero¹

Centro Andaluz de Biología del Desarrollo and Departamento de Ciencias Ambientales, Universidad Pablo de Olavide, 41013 Seville, Spain,¹ and Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom²

Received 9 June 2003/Accepted 8 September 2003

Pseudomonas sp. strain ADP uses the herbicide atrazine as the sole nitrogen source. We have devised a simple atrazine degradation assay to determine the effect of other nitrogen sources on the atrazine degradation pathway. The atrazine degradation rate was greatly decreased in cells grown on nitrogen sources that support rapid growth of *Pseudomonas* sp. strain ADP compared to cells cultivated on growth-limiting nitrogen sources. The presence of atrazine in addition to the nitrogen sources did not stimulate degradation. High degradation rates obtained in the presence of ammonium plus the glutamine synthetase inhibitor MSX and also with an *Nas*[−] mutant derivative grown on nitrate suggest that nitrogen regulation operates by sensing intracellular levels of some key nitrogen-containing metabolite. Nitrate amendment in soil microcosms resulted in decreased atrazine mineralization by the wild-type strain but not by the *Nas*[−] mutant. This suggests that, although nitrogen repression of the atrazine catabolic pathway may have a strong impact on atrazine biodegradation in nitrogen-fertilized soils, the use of selected mutant variants may contribute to overcoming this limitation.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a herbicide of the *s*-triazine family used for broad-leaf weed control in both crop and noncrop lands. Its widespread use and high mobility in soil have led to its frequent detection in surface water and groundwater at concentrations exceeding the maximum levels allowed (21, 22, 30, 37). The high incidence of atrazine-contaminated water and the increasing concern about the toxicological and ecotoxicological properties of atrazine (3, 6, 16, 17) have boosted research directed toward bioremediation of atrazine-polluted sites.

A few laboratories have reported the isolation of bacteria with the ability to utilize atrazine, achieving in some cases the complete mineralization of the herbicide (see reference 29 and references therein). The best-characterized atrazine-mineralizing bacterial strain is *Pseudomonas* sp. strain ADP (23), which uses atrazine as the sole nitrogen source by means of a catabolic pathway comprising six enzymatic steps (25, 40). The complete degradative pathway is encoded in the 108-kbp conjugative catabolic plasmid pADP-1, which was recently sequenced (25). The *atzA*, *atzB*, and *atzC* genes, responsible for the conversion of atrazine to cyanuric acid, are harbored at three distant positions within a large (>40 kbp) unstable region in pADP-1. Loss of one or more of these genes is the cause of the frequent appearance of *Atr*[−] (unable to utilize atrazine) mutants in nonselective medium (10). The genes involved in the *s*-triazine ring cleavage and ammonium release are clustered at a different location in pADP-1, to form the *atzDEF* operon (25). The *atzA*, *atzB*, and *atzC* genes have been shown to be widespread and plasmid borne in a number of

independent isolates from different parts of the world (9, 10, 31, 39, 40).

The influence of nitrogen compounds on the efficiency of atrazine catabolism has been the focus of a number of studies, since most atrazine-degrading bacteria use it as a nitrogen source and agricultural soils are often rich in nitrogen due to routine fertilization. Nitrogen amendments have been shown to have a negative effect on atrazine biodegradation by indigenous populations in soils (1, 2, 4, 12). The effect of nitrogen sources on atrazine degradation has also been tested in pure cultures of degrading organisms, and both nitrogen-repressed and nonresponsive strains have been described (15, 28, 36). *Pseudomonas* sp. strain ADP has been shown to metabolize atrazine rapidly when previously grown on atrazine or glycine while degradation was significantly slower with cells grown on ammonium, nitrate, or urea (5, 19).

In this paper, we use a simple atrazine degradation assay in resting cell suspensions to characterize nitrogen control of the atrazine catabolic pathway in *Pseudomonas* sp. strain ADP. In addition, we determine the effect of nitrate amendment in soil on atrazine mineralization by *Pseudomonas* sp. strain ADP and describe a mutant that, by overriding nitrogen control, mineralizes atrazine efficiently in nitrate-amended soil.

MATERIALS AND METHODS

Bacterial strains and growth media. Minimal medium for *Pseudomonas* sp. strain ADP was previously described (24). Sodium succinate (1 g liter^{−1}) was always used as a carbon source. Nitrogen sources were added at a final concentration of 4 mM nitrogen, except for atrazine, which was supplied at the saturating concentration from a reservoir. Briefly, 0.15 ml of a 20-mg liter^{−1} atrazine solution in methanol was added to the cap of a microcentrifuge tube. The open side of the cap was topped with a piece of cellulose dialysis membrane (Sigma-Aldrich Chemie, Steinheim, Germany), which was sealed in place with the top ring of the same microcentrifuge tube. The sealed reservoir was added to the water in the medium and sterilized by autoclaving. This method provides a continuous supply of atrazine without the problems derived from its low water solubility (31 mg liter^{−1}). Minimal agar plates were prepared as described above

* Corresponding author. Mailing address: Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide, Carretera de Utrera, Km. 1, 41013 Seville, Spain. Phone: 34-95-4349052. Fax: 34-95-4349376. E-mail: fgovrom@dex.upo.es.

but with the addition of 20 g of agar noble (Difco, Detroit, Mich.) liter⁻¹. Minimal atrazine agar plates included 0.4 g of atrazine liter⁻¹, added from a 20-g liter⁻¹ solution in methanol. Precipitation of atrazine crystals gives these plates a turbid appearance. Luria-Bertani (LB) agar was used as rich solid medium and was supplemented with atrazine (0.4 g liter⁻¹) when required. Cells were grown at 30°C, and shaking (~180 rpm) was used to aerate liquid cultures.

Pseudomonas sp. strain ADP (23) was used as a model atrazine-degrading bacterium. This strain is naturally resistant to ampicillin (40 mg/liter). Spontaneous Atr⁻ (deficient in atrazine utilization) mutants were isolated by taking advantage of the intrinsic instability of the atrazine-degradative phenotype (10). *Pseudomonas* sp. strain ADP was grown to saturation in liquid LB medium containing 1 g of NH₄Cl liter⁻¹ and then subcultured in the same medium. After three serial dilutions, cells were plated on LB agar containing 0.4 g of crystallized atrazine liter⁻¹. Colonies not surrounded by clear halos were streaked on minimal agar plates containing cyanuric acid as the sole nitrogen source to test for the cyanuric acid utilization phenotype (Cya). Both Cya⁺ and Cya⁻ colonies were examined for the presence of atrazine catabolic genes by PCR and restriction analysis of the pADP-1 plasmid. Two derivatives, named MPO100 and MPO101, were chosen for further study. MPO100 is cured from pADP-1, as inferred from its Atr⁻ Cya⁺ phenotype, lack of amplification with specific *atzA*, *atzB*, and *atzC* primers (see reference 9 for primer sequences), absence of plasmid bands in plasmid preparations, and 100-fold-increased conjugation frequency as a recipient of IncP⁺ plasmids (incompatible with pADP-1). MPO101 is Atr⁺ Cya⁺, and PCR and restriction analysis revealed that it harbors a deletion derivative of pADP-1 that lacks virtually the entire unstable region, including the *atzA*, *atzB*, and *atzC* genes and intervening sequences. A spontaneous Nas⁻ (deficient in nitrate assimilation) mutant derivative, named MPO102, was isolated based on chlorate resistance (14) by plating *Pseudomonas* sp. strain ADP on minimal medium containing KClO₃ (10 g liter⁻¹) and then scoring for slow growth on minimal agar plates containing nitrate as the sole nitrogen source. Spontaneous rifampin-resistant (Rif^r) derivatives of these strains were isolated when required by mass plating on LB agar containing rifampin (20 mg liter⁻¹).

Plasmid pADP-1 DNA preparation and manipulation. Small-scale plasmid preparations from *Pseudomonas* sp. strain ADP were performed by a simple alkaline lysis method, followed by one phenol-chloroform (24:1, vol/vol) extraction and ethanol precipitation (32). Preparations displayed one single slow-migrating band when run on 0.8% agarose gels. Digestion with *Bam*HI, *Eco*RV, or *Hind*III yielded band patterns consistent with those expected for pADP-1 according to the published sequence (25).

Resting cell assay of atrazine degradation. A simple resting cell assay was devised to measure the atrazine degradation of *Pseudomonas* sp. strain ADP based on a spectrophotometric atrazine chlorohydrolase assay described previously (7). Preinocula were grown to saturation by shaking overnight at 30°C in 3 ml of minimal medium, with ammonium as the sole nitrogen source. Cells were harvested by centrifugation, washed three times with phosphate-buffered saline solution (60 mM sodium-potassium phosphate [pH 7.0], 0.5 g of NaCl liter⁻¹), and resuspended in minimal medium with NH₄Cl, NaNO₃, L-proline, urea, L-serine, biuret, cyanuric acid, or atrazine as a nitrogen source. In some experiments, atrazine was added as a second nitrogen source. Cultures were subsequently shaken at 30°C for 16 to 20 h to early exponential phase (optical density at 600 nm [OD₆₀₀], ~0.3). Cells were harvested by centrifugation, and pellets were washed three times with U buffer (10 mM sodium phosphate [pH 7], 0.1 mM MgSO₄) and resuspended in the same buffer to an OD₆₀₀ of 0.25. Aliquots (5 ml) were placed in a water bath at 30°C and incubated for 2 min, and then 50 µl of 6 mM atrazine was added. Samples (0.5 ml) were withdrawn at 5-min intervals and centrifuged immediately for 3 min in a microcentrifuge at full speed. The atrazine concentration was determined from the absorbance at 225 nm (*A*₂₂₅) of the supernatants by using an experimentally derived molar extinction coefficient of 2.71 × 10⁴ liter cm⁻¹ mol⁻¹.

Effect of MSX on atrazine degradation. The glutamine synthetase inhibitor L-methionine sulfoximine (MSX) was used to inhibit nitrogen assimilation. To determine the appropriate concentration of MSX, *Pseudomonas* sp. strain ADP was grown in minimal medium with ammonium (1 g of NH₄Cl liter⁻¹) as the sole nitrogen source to an OD₆₀₀ of ~0.2. Then the culture was split between five flasks and exposed to 0, 0.2, 0.5, 0.8, or 1 mM MSX. The effect of the inhibitor on bacterial growth was determined by monitoring the OD₆₀₀ of the cultures.

To test the effect of MSX on atrazine degradation, *Pseudomonas* sp. strain ADP was grown as described above to an OD₆₀₀ of 0.2. Then the culture was split between two flasks, and MSX was added to one of them to a final concentration of 0.5 mM. After 6 h of incubation, cells were harvested and resting cell assays of atrazine degradation were performed.

Atrazine mineralization assay in soil microcosms. Microcosm assays of atrazine mineralization in soil were performed by using 25-ml airtight glass vials

containing 5 g (equivalent dry weight) of field-moist sieved (<2.8-mm pore size) agricultural surface soil (38). This soil has a low nitrate content (0.016 mg of nitrogen g of soil⁻¹), as determined by a direct colorimetric method (13). When required, soil was amended with KNO₃ (0.5 mg of nitrogen g of soil⁻¹). Spontaneous Rif^r derivatives of the *Pseudomonas* sp. strain ADP wild-type and MPO102 strains were grown to mid-exponential phase in minimal medium with atrazine as the sole nitrogen source. Cells were harvested, washed three times in sterile phosphate-buffered saline solution, and inoculated at a density of 10⁸ CFU g of soil⁻¹. Noninoculated controls received the same volume of autoclaved cell suspension. Water content was adjusted to 60% of the holding capacity of the soil. Uniformly ring-labeled [¹⁴C]atrazine was subsequently added to the soil (40 µg of atrazine g of soil⁻¹, 250 Bq g of soil⁻¹). A test tube containing 1 ml of 1 M NaOH was placed inside each vial before sealing. Soil microcosms were incubated at 25°C. In acclimation experiments, atrazine-grown *Pseudomonas* sp. strain ADP cells were incubated in the soil for 6 days prior to the addition of atrazine. At defined time intervals, the vials were opened, and the amount of ¹⁴CO₂ evolved was determined by scintillation counting of the NaOH solution. Before resealing and further incubation, fresh NaOH (1 ml) was added to each test tube. Scintillation was performed by using a Beckman LS6000TA liquid scintillation system. To monitor the change in bacterial numbers, a parallel set of microcosms was set up, but radiolabeled atrazine was replaced with cold atrazine. Viable *Pseudomonas* sp. strain ADP counts were determined by plating dilutions at selected time points on LB plates containing 20 mg of ampicillin liter⁻¹ and 10 mg of rifampin liter⁻¹.

Chemicals. Uniformly ring-labeled atrazine (7.8 mCi/mmol), cyanuric acid, biuret, urea, and MSX were purchased from Sigma-Aldrich-Riedel de Haën. Technical grade atrazine (>98% purity) was a gift from Novartis (Greensboro, N.C.). All other chemicals used were of reagent grade or better.

RESULTS

A simple assay to measure atrazine degradation by *Pseudomonas* sp. strain ADP. We have devised a simple method to measure atrazine degradation by resting cells of *Pseudomonas* sp. strain ADP. Our assay monitors the evolution of atrazine concentration in cell supernatants by means of *A*₂₂₅ measurements (see Materials and Methods for further details). Several control experiments were performed to test the validity of this method. A decrease in *A*₂₂₅ was not observed when (i) wild-type atrazine-grown cells were killed by boiling prior to the addition of atrazine; (ii) a *Pseudomonas* sp. strain ADP derivative (MPO100) cured for the pADP-1 plasmid grown on serine as the sole nitrogen source was used; and (iii) another mutant derivative (MPO101) that lacks the *atzA*, *atzB*, and *atzC* genes in pADP-1 grown on serine or cyanuric acid as the sole nitrogen source was used (data not shown). Living cells of wild-type *Pseudomonas* sp. strain ADP caused a significant decrease in *A*₂₂₅ under all of these conditions (see below). These results indicate that the decrease in *A*₂₂₅ correlates with the activity of plasmid-borne genes involved in atrazine catabolism.

Effect of nitrogen sources on atrazine degradation. We have used the resting cell assay to address the effect of growth on different nitrogen sources on atrazine degradation by *Pseudomonas* sp. strain ADP. As expected, atrazine-grown cells exhibited considerable atrazine degradation in our assay (Fig. 1). Cells grown on ammonium as the sole nitrogen source failed to reduce the atrazine concentration in the supernatant significantly (Fig. 1A). This was not a short-term effect of ammonium on atrazine transport or atrazine metabolizing enzymes, since addition of ammonium to the assay buffer did not affect atrazine degradation by atrazine-grown cells (data not shown). The presence of atrazine in addition to ammonium in the growth medium did not stimulate atrazine degradation, suggesting that ammonium-mediated repression operates regard-

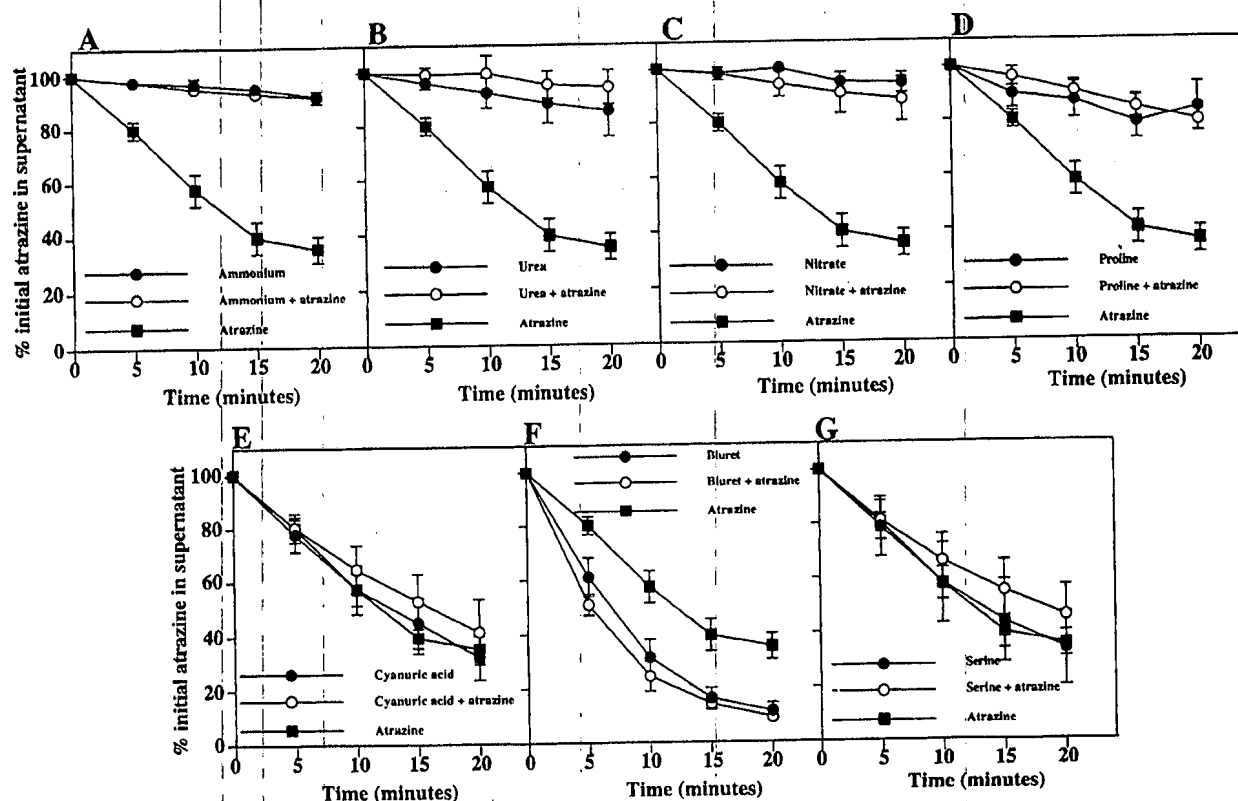


FIG. 1. Atrazine degradation in resting cells of *Pseudomonas* sp. strain ADP grown on different nitrogen sources. The percentage of the initial atrazine concentration remaining in the supernatant at each time point is plotted against time. Each panel represents the results obtained with a different nitrogen source: (A) Ammonium; (B) urea; (C) nitrate; (D) proline; (E) cyanuric acid; (F) biuret; (G) serine. The plot obtained with atrazine-grown cells is displayed in all panels for reference. Closed circles, cells grown on each compound as the sole nitrogen source; open circles, cells grown on each compound plus atrazine as nitrogen sources; closed squares, cells grown on atrazine as the sole nitrogen source. Symbols represent the averages of the results from three to four independent experiments. Error bars represent standard errors of the averages.

less of the presence of the herbicide in the culture medium. The effect of ammonium was not due to the loss of the catabolic genes, since efficient atrazine degradation was evident when cultures were allowed to exhaust the ammonium by growing to stationary phase (data not shown). Several other nitrogen sources were tested with the same approach. The rate of atrazine degradation by cells grown on urea, proline, or nitrate was low regardless of the presence of atrazine in the growth medium (Fig. 1B to D). However, cyanuric acid, biuret, and serine failed to repress atrazine degradation both in the presence and in the absence of atrazine (Fig. 1E to G). Interestingly, nitrogen sources (ammonium, urea, and proline) that supported fast growth of *Pseudomonas* sp. strain ADP (doubling time, ~2 h) repressed atrazine degradation while degradation was efficient with those nitrogen sources on which *Pseudomonas* sp. strain ADP exhibited slower growth (doubling time, >6 h), such as biuret, atrazine, or serine. *Pseudomonas* sp. strain ADP had similar doubling times (~3 h) when grown on nitrate and cyanuric acid, but degradation was observed with cyanuric acid-grown cells and not with nitrate-grown cells. However, this may relate to a specific positive effect of cyanuric acid on the degradative pathway (V. García-González, unpublished data). Taken together, our results suggest that one or more steps in atrazine utilization are repressed under nitrogen-sufficient growth conditions and activated un-

der nitrogen limitation. Activation does not appear to require atrazine or any of its metabolites (Fig. 1G). In addition, atrazine does not stimulate its own catabolism when a repressing nitrogen source is present (Fig. 1A to D).

Nitrogen incorporation to carbon backbones is required for repression of atrazine degradation. To test whether nitrogen control of atrazine degradation requires assimilation of the nitrogen source, we used MSX, an inhibitor of glutamine synthetase. Treatment of cells growing on ammonium with sublethal concentrations of MSX has been shown to provoke nitrogen starvation in several bacterial species by slowing down ammonium incorporation into carbon backbones and depleting the internal pools of glutamine (8, 34). A concentration of 0.5 mM MSX was chosen, since it had a severe effect on the growth rate but did not cause a complete inhibition of bacterial growth (Fig. 2A). Atrazine degradation by ammonium-grown cells was greatly stimulated when 0.5 mM MSX was also present in the growth medium, as assessed by resting cell assays (Fig. 2B), indicating that slow assimilation is sufficient to override ammonium-mediated repression of the pathway.

As an alternative means to test nitrogen signaling in *Pseudomonas* sp. strain ADP, a genetic approach was used. A *Nas*⁻ mutant (MPO102) was isolated that grows slowly on nitrate but exhibits a growth rate similar to that of the wild type on ammonium (Fig. 3A) and other nitrogen sources (data not

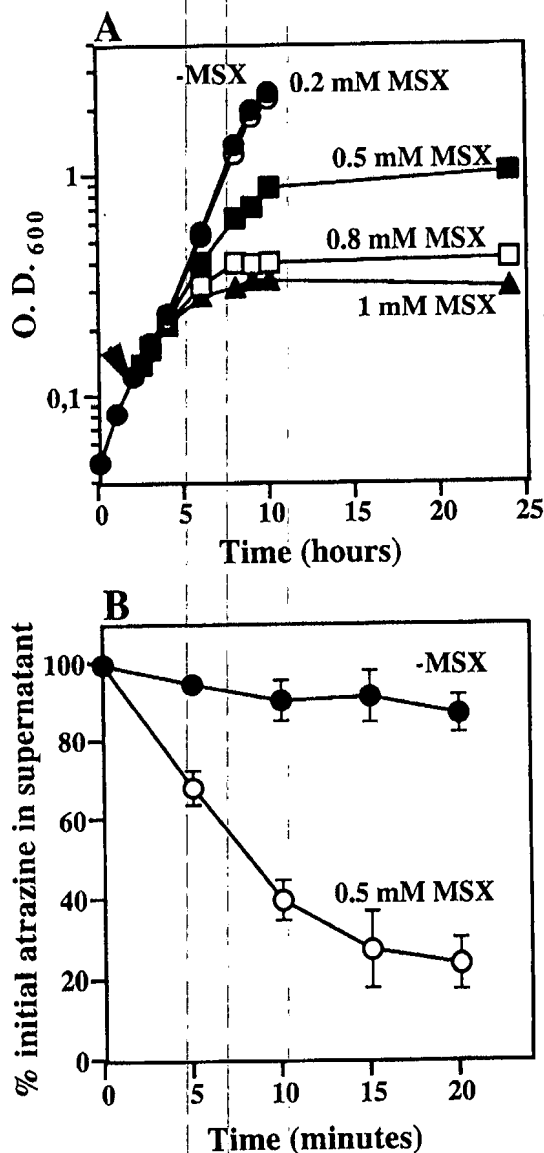


FIG. 2. Effect of MSX on growth and atrazine degradation by resting cells of *Pseudomonas* sp. strain ADP. (A) Growth curves of *Pseudomonas* sp. strain ADP growing on ammonium as the sole nitrogen source with the addition of 1 mM MSX (closed triangles), 0.8 mM MSX (open squares), 0.5 mM MSX (closed squares), 0.2 mM MSX (open circles), or no MSX (closed circles, -MSX). The arrowhead indicates the time of addition of MSX. (B) Resting cell assay of atrazine degradation by *Pseudomonas* sp. strain ADP grown on ammonium (closed circles, -MSX) or ammonium and 0.5 mM MSX (open circles). Symbols represent the averages of the results from four independent experiments. Error bars represent standard errors of the averages.

shown). Unlike the wild type, MPO102 degraded atrazine efficiently in resting cell assays when grown on nitrate alone or on nitrate and atrazine as nitrogen sources (compare Fig. 3B with 1C). These results strongly suggest that the signaling pathway responsible for nitrogen control of atrazine catabolism requires intracellular incorporation of nitrogen to carbon backbones. In addition, growth-limiting nitrogen assimilation

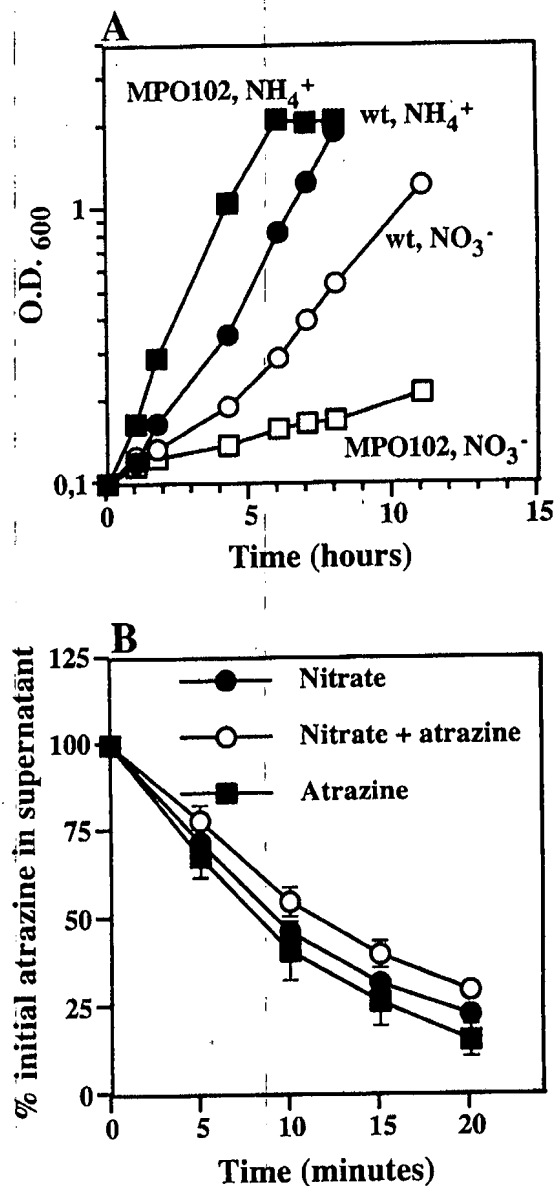


FIG. 3. Growth and atrazine degradation by resting cells of the *Nas*⁻ MPO102 strain. (A) Growth curve of wild-type (wt) *Pseudomonas* sp. strain ADP (circles) and MPO102 (squares) growing on ammonium (closed symbols) or nitrate (open symbols) as the sole nitrogen source. (B) Resting cell assay of atrazine degradation by MPO102 grown on nitrate (closed circles), nitrate and atrazine (open circles), and atrazine (closed squares) as nitrogen sources. Symbols represent the averages of the results from three to four independent experiments. Error bars represent standard errors of the averages.

is sufficient to induce atrazine degradation, regardless of the chemical nature of the nitrogen source tested.

Nitrate addition inhibits atrazine mineralization by *Pseudomonas* sp. strain ADP in soil. Nitrogen control of atrazine utilization may pose a limitation to degradation of the herbicide by *Pseudomonas* sp. strain ADP in nitrogen-rich agricultural soils. To address this issue, soil microcosm experiments were designed in which the rate of radiolabeled atrazine min-

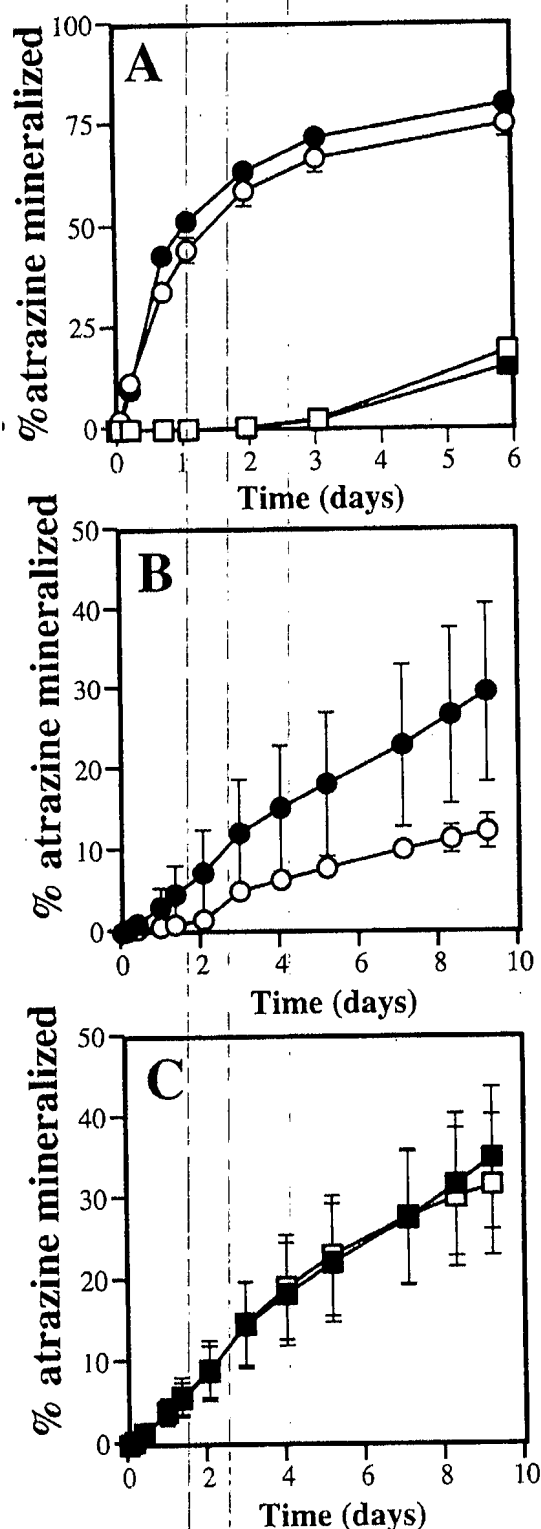


FIG. 4. Atrazine mineralization by *Pseudomonas* sp. strain ADP in soil microcosms. (A) Atrazine mineralization by wild-type *Pseudomonas* sp. strain ADP (closed symbols) and MPO102 (open symbols). Cells were grown on atrazine as the sole nitrogen source and were inoculated directly to unamended soil (circles) or heat killed prior to addition (squares). (B) Atrazine mineralization by wild-type *Pseudomonas* sp. strain ADP after 6 days of adaptation to unamended (closed circles) or nitrate-amended ($0.5 \text{ mg of NO}_3\text{N g}^{-1}$) soil (open circles).

eralization by *Pseudomonas* sp. strain ADP was determined in nonsterile low-nitrogen soil samples. In initial control experiments, the addition of wild-type atrazine-grown *Pseudomonas* sp. strain ADP to unamended soil greatly stimulated atrazine mineralization compared to a control to which heat-killed cells were added (Fig. 4A). Interestingly, a low level of atrazine mineralization was evident in the control vials, indicating that atrazine-mineralizing organisms were part of the natural flora in the soil. Atrazine mineralization by *Pseudomonas* sp. strain ADP was rapid, with more than 50% of the label released within the first 24 h after atrazine addition. Similar results were obtained with the MPO102 mutant (Fig. 4A). To determine the effect of nitrate on atrazine mineralization in soil, *Pseudomonas* sp. strain ADP cells were inoculated into nitrate-amended and unamended soil microcosms and allowed to acclimate to the soil environment for 6 days prior to the addition of atrazine (Fig. 4, B). Atrazine mineralization by *Pseudomonas* sp. strain ADP in the amended soil was decreased threefold compared to the unamended control, indicating that atrazine mineralization in soil is repressed by nitrate. Mineralization rates were lower than in the previous experiment, likely due to the decrease in the *Pseudomonas* sp. strain ADP population in the soil during the acclimation period (half-life, ~ 3.1 days).

Atrazine mineralization by the MPO102 mutant is not repressed by added nitrate. To test whether atrazine degradation by the MPO102 mutant is affected by the presence of nitrate in a soil environment, atrazine-grown MPO102 was also acclimated for 6 days in unamended and nitrate-amended soil microcosms. Radiolabeled atrazine was then added, and mineralization was monitored (Fig. 4C). The atrazine mineralization rate in the unamended soil was similar to that observed with wild-type *Pseudomonas* sp. strain ADP (compare unamended controls in Fig. 4B and C). Interestingly, there was no significant difference between mineralization rates in unamended and nitrate-amended soils when MPO102 was used. This difference between the wild-type and mutant strains is not due to increased survival of MPO102, since its half-life in nitrate-amended soil was slightly lower than that of the wild-type strain (2.3 days). These results indicate that atrazine degradation by MPO102 is not repressed by nitrate in the soil.

DISCUSSION

In this study, we have found that the presence of preferential nitrogen sources in the environment is detrimental to atrazine degradation by *Pseudomonas* sp. strain ADP. These results confirm and extend the observations of Bichat et al. (5). These authors noted that atrazine degradation by cells previously grown on ammonium, nitrate, or urea as the sole nitrogen source was slow compared to atrazine- or glycine-grown cells. Our data clearly show that certain nitrogen sources have an inhibitory effect on the catabolic pathway that is not prevented

(C) Atrazine mineralization by MPO102 after 6 days of adaptation to unamended (closed squares) or nitrate-amended (open squares) soil. Symbols represent the averages of the results from three independent experiments. Error bars represent standard errors of the averages.

by the simultaneous presence of atrazine in the medium. This and the fact that efficient atrazine degradation can be achieved with compounds unrelated to the atrazine degradative pathway, i.e., serine (Fig. 1G) or glycine (5) as sole nitrogen sources indicate that neither atrazine nor any of its metabolites are required for induction of the pathway.

General nitrogen control is a well-characterized regulatory phenomenon in a number of gram-positive and gram-negative organisms (26). Our results suggest that atrazine degradation may be subjected to general nitrogen control resembling that described in enterobacteria: (i) regulation is likely to occur primarily at the gene expression level, since addition of ammonium to the assay buffer had no inhibitory effect on atrazine removal by atrazine-grown cells; (ii) atrazine catabolism is repressed in the presence of nitrogen sources that support fast growth of the strain, suggesting preferential nitrogen assimilation from these nitrogen sources; and (iii) nitrogen status is sensed from changes in intracellular pools of metabolites, rather than from the nitrogen species present in the medium (11, 18, 33). Nitrogen control in the enterobacteria requires an alternative form of RNA polymerase holoenzyme that contains σ^{54} (the product of *rpoN*) (26). Interestingly, an *rpoN* mutant of *Pseudomonas putida* KT2440 can grow normally on the atrazine degradation-repressing nitrogen sources ammonium, urea, and proline but fails to grow on the nonrepressing nitrogen sources serine and glycine (20). The only exception to this correlation is nitrate, which cannot be utilized as a nitrogen source by the *P. putida rpoN* mutant but represses atrazine degradation in *Pseudomonas* sp. strain ADP. However, comparison of the growth rates of the two organisms suggests that nitrate may be a growth-limiting nitrogen source for *P. putida* KT2440 but not for *Pseudomonas* sp. strain ADP. It is tempting to speculate that a conserved σ^{54} -dependent mechanism is responsible for general nitrogen control in the enterobacteria and the pseudomonads. In that regard, it is worth noting that genes homologous to the signal transduction and regulatory elements for general nitrogen control in the enterobacteria have been found in the sequenced genomes of *Pseudomonas aeruginosa* PAO1 and *P. putida* KT2440 (27, 35).

Many xenobiotic-degrading bacterial strains that perform well in laboratory media turn out to be poor degraders in a natural setting (42). One possible explanation for this phenomenon is the presence of environmental traits that have a negative impact on the expression of the degradative pathway. Our results indicate that nitrogen control of atrazine metabolism is functional under soil conditions and may therefore limit the potential of *Pseudomonas* sp. strain ADP for atrazine bioremediation in nitrogen-fertilized agricultural soils. On the other hand, the absence of nitrate repression in the MPO102 mutant and the fact that nitrate is the major nitrogen species in well-aerated soils (41) suggest that MPO102 may be a more suitable strain for bioremediation of atrazine-contaminated agricultural soils with a high nitrogen content.

ACKNOWLEDGMENTS

We thank Inés Canosa for critical review of the manuscript.

This work was supported by the European Union project QLK3-CT-1999-00041 and by a fellowship of the F.P.U. program of the Spanish Ministerio de Educación y Cultura awarded to V.G.-G.

REFERENCES

1. Abdelhafid, R., S. Houot, and E. Barriuso. 2000. Dependence of atrazine degradation on C and N availability in adapted and non-adapted soils. *Soil Biol. Biochem.* 32:389–401.
2. Abdelhafid, R., S. Houot, and E. Barriuso. 2000. How increasing availabilities of carbon and nitrogen affect atrazine behaviour in soils. *Biol. Fertil. Soils* 30:333–340.
3. Allran, J. W., and W. H. Karasov. 2000. Effects of atrazine and nitrate on the northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environ. Toxicol. Chem.* 19:2850–2855.
4. Alvey, S., and D. E. Crowley. 1995. Influence of organic amendments on biodegradation of atrazine as a nitrogen source. *J. Environ. Qual.* 24:1156–1162.
5. Bichat, F., G. K. Sims, and R. L. Mulvaney. 1999. Microbial utilization of heterocyclic nitrogen from atrazine. *Soil Sci. Soc. Am. J.* 63:100–110.
6. Biradar, D. P., and A. L. Rayburn. 1995. Chromosomal damage induced by herbicide contamination at concentrations observed in public water supplies. *J. Environ. Qual.* 24:1222–1225.
7. Bouquard, C., J. Ouazzani, J.-C. Prom, Y. Michel-Briand, and P. Plésiat. 1997. Dechlorination of atrazine by a *Rhizobium* sp. isolate. *Appl. Environ. Microbiol.* 63:916–923.
8. Brencley, J. E. 1973. Effect of methionine sulfoximine and methionine sulfone on glutamate synthesis in *Klebsiella aerogenes*. *J. Bacteriol.* 114:666–673.
9. de Souza, M. L., J. Seffernick, B. Martinez, M. J. Sadowsky, and L. P. Wackett. 1998. The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J. Bacteriol.* 180:1951–1954.
10. de Souza, M. L., L. P. Wackett, and M. J. Sadowsky. 1998. The *atzABC* genes encoding atrazine catabolism are located on a self-transmissible plasmid in *Pseudomonas* sp. strain ADP. *Appl. Environ. Microbiol.* 64:2323–2326.
11. Engleman, E. G., and S. H. Francis. 1978. Cascade control of *E. coli* glutamine synthetase. II. Metabolite regulation of the enzymes in the cascade. *Arch. Biochem. Biophys.* 191:602–612.
12. Entry, J. A., K. G. Mattson, and W. H. Emmingham. 1993. The influence of nitrogen on atrazine and 2, 4-dichlorophenoxyacetic acid mineralization in grassland soils. *Biol. Fertil. Soils* 16:179–182.
13. Foster, J. C. 1995. Soil nitrogen, p. 79–87. In K. Alef and P. Nannipieri (ed.), *Methods in applied soil microbiology and biochemistry*. Academic Press, New York, N.Y.
14. Garzón, A., J. Li, A. Flores, J. Casadesús, and V. Stewart. 1992. Molybdenum cofactor (chlorate-resistant) mutants of *Klebsiella pneumoniae* M5a1 can use hypoxanthine as the sole nitrogen source. *J. Bacteriol.* 174:6298–6302.
15. Gebendinger, N., and M. Radosevich. 1999. Inhibition of atrazine degradation by cyanazine and exogenous nitrogen in bacterial isolate M91–3. *Appl. Microbiol. Biotechnol.* 51:375–381.
16. Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffle, and A. Vonk. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. *Environ. Health Perspect.* 111:568–575.
17. Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. USA* 99:5476–5480.
18. Ikeda, T. P., A. E. Shauger, and S. Kustu. 1996. *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. *J. Mol. Biol.* 259:589–607.
19. Katz, I., C. G. Dosoretz, R. T. Mandelbaum, and M. Green. 2001. Atrazine degradation under denitrifying conditions in continuous culture of *Pseudomonas* ADP. *Water Res.* 35:3272–3275.
20. Köhler, T., S. Harayama, J. L. Ramos, and K. N. Timmis. 1989. Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. *J. Bacteriol.* 171:4326–4333.
21. Kolpin, D. W., and S. J. Kalkhoff. 1993. Atrazine degradation in a small stream in Iowa. *Environ. Sci. Technol.* 27:134–139.
22. Kolpin, D. W., E. M. Thurman, and D. A. Goolsby. 1996. Occurrence of selected herbicides and their metabolites in near-surface aquifers of the midwestern United States. *Environ. Sci. Technol.* 30:385–390.
23. Mandelbaum, R. T., L. P. Wackett, and D. L. Allan. 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Appl. Environ. Microbiol.* 61:1451–1457.
24. Mandelbaum, R. T., L. P. Wackett, and D. L. Allan. 1993. Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. *Appl. Environ. Microbiol.* 59:1695–1701.
25. Martinez, B., J. Tomkins, L. P. Wackett, R. Wing, and M. J. Sadowsky. 2001. Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J. Bacteriol.* 183:5684–5697.
26. Merrick, M. J., and R. A. Edwards. 1995. Nitrogen control in bacteria. *Microbiol. Rev.* 59:604–622.
27. Nelson, K. E., C. Weinl, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac,

- M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. C. Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4:799–808.
28. Rndosevich, M., S. J. Traina, Y. L. Hao, and O. H. Tuovinen. 1995. Degradation and mineralization of atrazine by a soil bacterial isolate. *Appl. Environ. Microbiol.* 61:297–302.
29. Ralebitso, T. K., E. Senior, and H. W. van Versveld. 2002. Microbial aspects of atrazine degradation in natural environments. *Biodegradation* 13:11–19.
30. Richards, R. P., and D. B. Baker. 1993. Pesticide concentration patterns in agricultural drainage networks in the Lake Erie basin. *Environ. Toxicol. Chem.* 12:13–36.
31. Rousseaux, S., A. Hartmann, and G. Soulas. 2001. Isolation and characterization of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiol. Ecol.* 36:211–222.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. Senior, P. J. 1975. Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: studies with the continuous-culture technique. *J. Bacteriol.* 123:407–418.
34. Smith, C. J., R. B. Hespell, and M. P. Bryant. 1981. Regulation of urease and ammonia assimilatory enzymes in *Selenomonas ruminantium*. *Appl. Environ. Microbiol.* 42:89–96.
35. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrenner, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964.
36. Struthers, J. K., K. Jayachandran, and T. B. Moorman. 1998. Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *Appl. Environ. Microbiol.* 64:3368–3375.
37. Tappe, W., J. Groeneweg, and B. Jantsch. 2002. Diffuse atrazine pollution in German aquifers. *Biodegradation* 13:3–10.
38. Taylor, J. P., B. Wilson, M. S. Mills, and R. G. Burns. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol. Biochem.* 34:387–401.
39. Topp, E., H. Zhu, S. M. Nour, S. Houot, M. Lewis, and D. Cuppels. 2000. Characterization of an atrazine-degrading *Pseudaminobacter* sp. isolated from Canadian and French agricultural soils. *Appl. Environ. Microbiol.* 66:2773–2782.
40. Wackett, L. P., M. J. Sadowsky, B. Martinez, and N. Shapir. 2002. Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. *Appl. Microbiol. Biotechnol.* 58:39–45.
41. Wild, A. 1988. Plant nutrients in soil: nitrogen, p. 652–695. In A. Wild (ed.), *Russell's soil conditions and plant growth*. Longman, Harlow, Essex, United Kingdom.
42. Young, C. S., and R. G. Burns. 1993. Detection, survival and activity of bacteria added to soil, p. 1–63. In J.-M. Bollag and G. Stotzky (ed.), *Soil biochemistry*, vol. 8. Marcel Dekker, New York, N.Y.

Isolation and characterization of a novel simazine-degrading bacterium from agricultural soil of central Chile, *Pseudomonas* sp. MHP41

Marcela Hernández^{1,2}, Patricio Villalobos¹, Verónica Morgante¹, Myriam González¹, Caroline Reiff³, Edward Moore^{3,4} & Michael Seeger¹

¹Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Millennium Nucleus of Microbial Ecology and Environmental Microbiology and Biotechnology EMBA, Departamento de Química, Universidad Técnica Federico Santa María, Valparaíso, Chile; ²Programa Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Temuco, Chile; ³Division of Environmental Sciences, The Macaulay Institute, Aberdeen, Scotland, UK; and ⁴CCUG – Department of Clinical Bacteriology, Sahlgrenska Academy, Göteborg University, Göteborg, Sweden

Correspondence: Michael Seeger, Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Millennium Nucleus of Microbial Ecology and Environmental Microbiology and Biotechnology EMBA, Departamento de Química, Universidad Técnica Federico Santa María, Avenida España 1680, Valparaíso, Chile. Tel.: +56 32 2654236; fax: +56 32 2654782; e-mail: michael.seeger@usm.cl

Received 18 April 2008; accepted 16 June 2008.

First published online 18 July 2008.

DOI:10.1111/j.1574-6968.2008.01274.x

Editor: Aharon Oren

Keywords

simazine; *atz* genes; biodegradation; *Pseudomonas* sp. strain MHP41; 16S rRNA gene sequence.

Abstract

s-Triazine herbicides are used extensively in South America in agriculture and forestry. In this study, a bacterium designated as strain MHP41, capable of degrading simazine and atrazine, was isolated from agricultural soil in the Quillota valley, central Chile. Strain MHP41 is able to grow in minimal medium, using simazine as the sole nitrogen source. In this medium, the bacterium exhibited a growth rate of $\mu = 0.10 \text{ h}^{-1}$, yielding a high biomass of $4.2 \times 10^8 \text{ CFU mL}^{-1}$. Resting cells of strain MHP41 degrade more than 80% of simazine within 60 min. The *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes encoding the enzymes of the simazine upper and lower pathways were detected in strain MHP41. The motile Gram-negative bacterium was identified as a *Pseudomonas* sp., based on the Biolog microplate system and comparative sequence analyses of the 16S rRNA gene. Amplified ribosomal DNA restriction analysis allowed the differentiation of strain MHP41 from *Pseudomonas* sp. ADP. The comparative 16S rRNA gene sequence analyses suggested that strain MHP41 is closely related to *Pseudomonas nitroreducens* and *Pseudomonas multiresinovorans*. This is the first *s*-triazine-degrading bacterium isolated in South America. Strain MHP41 is a potential biocatalyst for the remediation of *s*-triazine-contaminated environments.

Introduction

s-Triazine herbicides, such as simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine], have been used extensively for the control of weeds in many regions of the world (Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995; Santiago-Mora *et al.*, 2005). These agrochemicals are used mainly as pre- and postemergence herbicides. They are strong inhibitors of photosynthesis, interrupting the electron transport chain in photosystem II (Foth, 1999; Rousseaux *et al.*, 2001; Tappe *et al.*, 2002). The mobility of *s*-triazine in soil has contributed to the contamination of surface- and groundwater. *s*-Triazines have been detected in drinking water, exceeding

the standard acceptable levels of 0.1 and $3.0 \mu\text{g L}^{-1}$ allowed by the European Union and USA regulatory authorities, respectively (Mandelbaum *et al.*, 1995; Rousseaux *et al.*, 2001). Atrazine has been classified as a class C carcinogen (Ralebitso *et al.*, 2002; Tappe *et al.*, 2002). Simazine and atrazine are the herbicides applied most frequently in Chile, where 350 tonnes were sold in 2004 (Dinamarca *et al.*, 2007).

s-Triazine herbicides persist in soils, as well as in aquifers. Chemical and biological processes are involved in the dissipation of atrazine and simazine in soil (Gunasekara *et al.*, 2007). However, microbial degradation is probably the main mechanism for removing *s*-triazine from soils (Ralebitso *et al.*, 2002). Bacteria able to degrade *s*-triazine

herbicides have been isolated (Mandelbaum *et al.*, 1995; Topp *et al.*, 2000a,b; Rousseaux *et al.*, 2001) and the *s*-triazine degradation pathways of *Pseudomonas* sp. ADP have been characterized (Martínez *et al.*, 2001). The 'upper' degradation pathway for *s*-triazine catabolism to cyanuric acid is encoded by the *atzA*, *atzB* and *atzC* genes (De Souza *et al.*, 1998a). Cyanuric acid is mineralized to carbon dioxide and ammonia by three enzymes of the 'lower' degradation pathway, encoded by the *atzD*, *atzE* and *atzF* genes (Strong *et al.*, 2002).

Here, we present the report of a novel simazine-degrading bacterium isolated from agricultural soil of central Chile, *Pseudomonas* sp. strain MHP41, which is able to efficiently degrade simazine, to use it for growth as the sole nitrogen source and could represent an important biocatalyst for the remediation of *s*-triazine-contaminated environments.

Materials and methods

Materials

Commercial simazine (Gesatop WP90) used for selective enrichment cultivation was purchased from Syngenta (Greensboro, United States). Simazine (99% pure) and standard simazine (> 99% pure) were purchased from Atanor (Buenos Aires, Argentina) and Dr Ehrenstorfer GmbH (Augsburg, Germany), respectively.

Soil sampling

Soils were sampled from an avocado agricultural field located in the Quillota valley, central Chile. These soils have been subjected to continuous simazine application for more than 20 years. Surface soil samples (0–20 cm) were collected adjacent to 10 randomly selected avocado trees in December 2002, air-dried and stored at 4 °C until analysed.

Enrichment and bacterial isolation

Bacterial strains were isolated by selective enrichment. Ten grams of soil (dry weight) was added to 90 mL of AM medium (AM) (Rousseaux *et al.*, 2001) with added simazine as the sole nitrogen source. The medium contained (L^{-1}): 1.6 g K_2HPO_4 ; 0.4 g KH_2PO_4 ; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; and 30 mg simazine. The medium pH was adjusted to 7.0 and, after autoclaving, the medium was supplemented with filter-sterilized solutions of sodium citrate ($10 mL L^{-1}$ of a $100 g L^{-1}$ solution) as a carbon source; trace elements ($1 mL L^{-1}$ of a solution containing $2 g L^{-1}$ boric acid; $1.8 g L^{-1}$ $MnSO_4 \cdot H_2O$; $0.2 g L^{-1}$ $ZnSO_4 \cdot 7H_2O$; $0.1 g L^{-1}$ $CuSO_4 \cdot 5H_2O$; and $0.25 g L^{-1}$ $NaMoO_4 \cdot 2H_2O$); vitamins ($1 mL L^{-1}$ of a solution containing $100 mg L^{-1}$ of thiamine-HCl and $40 mg L^{-1}$ of biotin); and $FeSO_4 \cdot 7H_2O$ ($1 mL L^{-1}$ of a $5 g L^{-1}$ solution). Cycloheximide ($50 mg L^{-1}$) was included in the medium to inhibit eukaryotic cell growth (Rousseaux

et al., 2001). Enrichments were incubated at 28 °C with agitation (150 r.p.m.). Enrichment cultures were subcultured on the same medium at 2-week intervals. Isolates were obtained by spread plating on AM agar with simazine (2.5 mM) and subsequently purifying by streak plating. AM agar cultures were incubated at 28 °C. Strain MHP41 was deposited in the Culture Collection, University of Göteborg (CCUG), with the accession number CCUG 50378.

Bacterial growth

Bacteria were grown in AM broth, supplemented with simazine as the sole nitrogen source. Bacterial growth was analysed by measuring turbidity at 600 nm and by CFU counting. Strain MHP41 was cultivated on Tryptic Soy (TS) broth for determination of the optimal growth temperature measured between 5 and 42 °C. AM agar with simazine (3.0 mM) and TS agar with atrazine (2.5 mM) were used to observe clearing zones of bacterial colonies indicating degradation of *s*-triazines.

Simazine degradation by resting cell assays

Bacterial cultures were grown at 30 °C as described by García-González *et al.* (2003) to the exponential phase (turbidity_{600 nm} ~0.3). Cells were harvested by centrifugation and washed three times with a phosphate buffer saline solution [60 mM sodium phosphate (pH 7.0), $0.5 g L^{-1}$ NaCl]. Finally, cells were resuspended to a turbidity_{600 nm} of 0.25–0.30 using 10 mM sodium phosphate (pH 7.0), supplemented with 0.1 mM $MgSO_4$. Simazine was added to cell suspensions, to a final concentration of 0.06 mM, and incubated further at 30 °C. Aliquots of cell suspensions were taken after different incubation times and centrifuged for 1 min at 16 000 g. The simazine concentration was determined by measuring the $A_{225 nm}$ of the supernatants. Degradation assays were performed in triplicate.

Analyses of *atz* genes

The *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes were assessed using PCR, with specific primers as described (De Souza *et al.*, 1998b; Devers *et al.*, 2004). Genomic DNA was prepared from single colonies resuspended in 100 μL Tris-EDTA buffer, heated to 95 °C for 5 min and centrifuged briefly. The supernatant (2.0 μL) was used for PCR (25 μL final volume).

Substrate-oxidation analyses

Strain MHP41 was characterized, using the BIOLOG microplate system (Biolog Inc., Hayward, CA), according to the protocol of the manufacturer. The GN2 microplate was incubated for 24 h in the dark at 30 °C.

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA was performed on the bacterial strains MHP41 and *Pseudomonas* sp. ADP (Mandelbaum *et al.*, 1995). A bacterial colony was suspended in 100 μ L sterile deionized H₂O and heated at 94 °C for 10 min. The 16S rRNA genes were amplified by PCR, using bacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). PCR products were digested with two restriction endonucleases, MspI or HhaI (Fermentas, Ontario), according to the protocols of the manufacturer. ARDRA profiles (2–5 μ L of digested 16S rRNA gene products) were separated by electrophoresis (MiniProtein, BioRad) in an 8% polyacrylamide gel in Tris borate EDTA buffer, at 130 V, for 60 min, and stained with ethidium bromide.

16S rRNA gene sequence analysis

Genomic DNA was prepared using the FastDNA[®] Kit for bacteria and the protocols of the manufacturer (Bio 101 Systems, Q-BioGene). The 16S rRNA genes were amplified by PCR, in duplicate, using bacterial primers: 16F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and 23R458 (5'-CCCCTTCCCTCACGGTAC-3'), complementary to nucleotide positions 7–27 of the 16S rRNA gene and positions 458–476 of the 23S rRNA gene (*Escherichia coli* rRNA gene sequence numbering), respectively, and the protocols described previously (Hauben *et al.*, 1997). PCR products were sequenced directly, using the sequencing primers described previously (Hauben *et al.*, 1997) and the Taq DyeDeoxy Terminator Cycle Sequencing kit, version 3.1, an ABI model 3100 DNA Sequencer, according to the protocols of the manufacturer (Applied Biosystems Inc., Foster City, CA). Sequence data were edited manually and compared with reference 16S rRNA gene sequence data, including those of type strains of species with validly published names, using the FastA3 algorithm (<http://www.ebi.ac.uk/fasta3/>) (Pearson & Lipman, 1988).

Results

Isolation and characterization of a novel simazine-degrading bacterium

Bacterial strains able to grow on AM agar with simazine as the sole nitrogen source were isolated from soil samples from agricultural fields with long-term simazine treatments. One of these strains, obtained from the fourth subculture, was analysed in detail for its degradation capacity and designated as strain MHP41.

Strain MHP41 was a Gram-negative, motile bacterium that formed round, smooth and convex colonies on TS agar.

This bacterium was able to grow on TS broth at a wide range of temperatures, with an optimum temperature of c. 30 °C. Strain MHP41 was able to grow using simazine or atrazine as the sole nitrogen source. To visualize *s*-triazine degradation ability on agar plates, strain MHP41 was grown on AM agar plates supplemented with simazine (3.0 mM) as the sole nitrogen source and on plates of TS agar supplemented with atrazine (2.5 mM). The clearing zones observed around the colonies indicated *s*-triazine degradation.

The strain, grown in AM broth supplemented with citrate as a carbon source and simazine as the sole nitrogen source, at 30 °C, attained a turbidity of 1.03 at 600 nm and a biomass of 4.2×10^8 CFU mL⁻¹ (data not shown). In this medium, the bacterium exhibited a growth rate of $\mu = 0.10$ h⁻¹.

Resting cells of strain MHP41, cultivated on minimal medium with simazine as the only nitrogen source, were observed to degrade 80% of available simazine in 1 h (Fig. 1).

Detection of *atz* catabolic genes

The *atzA*, *atzB* and *atzC* genes, encoding the 'upper' *s*-triazine catabolic pathway, were detected by PCR amplification using a specific set of primers (De Souza *et al.*, 1998b). The expected sizes of PCR products were observed: *atzA*: 0.5 kb; *atzB*: 0.5 kb; and *atzC*: 0.6 kb (Fig. 2). Additionally, the *atzD*, *atzE* and *atzF* genes, encoding the 'lower' *s*-triazine catabolic pathway, were also detected in strain MHP41. The PCR products, *atzD*: 0.5 kb; *atzE*: 0.2 kb; and *atzF*: 0.3 kb *atzF*, are shown in Fig. 2.

Taxonomic characterization and identification

Strain MHP41 was determined to be a Gram-negative, motile, rod-shaped bacterium. It was catalase positive and oxidase negative. It used glucose and fructose as carbon sources but did not use sucrose, galactose, lactose or

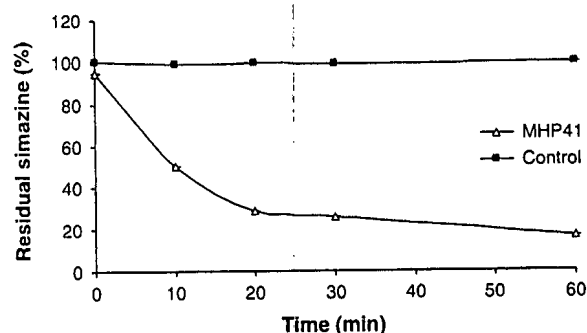


Fig. 1. Simazine degradation by *Pseudomonas* sp. MHP41. Resting cells of a culture previously grown in AM broth with simazine were incubated with simazine (0.06 mM). The control was phosphate buffer supplemented with MgSO₄. Each value represents an average of three independent experiments.

maltose. The isolate MHP41 was tentatively identified as a *Pseudomonas* sp. by the Biolog identification system, with a similarity index of 0.76 with *Pseudomonas nitroreducens* and *Pseudomonas azelaica* (> 0.50 is considered to be an acceptable match) (Table 1). ARDRA electrophoretic profiling (using restriction endonucleases MspI or HhaI) was used for comparing strain MHP41 with the atrazine-degrading strain *Pseudomonas* sp. ADP. ARDRA patterns showed important differences in the 16S rRNA gene sequences of the two organisms and allowed the differentiation of strain MHP41 from *Pseudomonas* sp. ADP (Fig. 3).

Comparative 16S rRNA gene sequence analysis identified strain MHP41 as a species of *Pseudomonas*, with a sequence most similar to those of *Pseudomonas multiresinovorans* (99.8%) and *P. nitroreducens* (99.7%) (Table 2). The 16S rRNA gene sequence of strain MHP41 was submitted to the EMBL Nucleotide Sequence Database (Kulikova *et al.*, 2007), under the accession number AM922106.

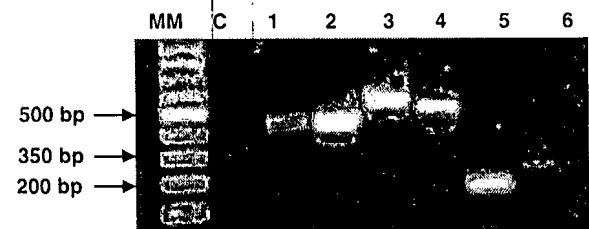


Fig. 2. Detection by PCR of *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes for simazine degradation in *Pseudomonas* sp. MHP41. PCR-amplification products: *atzA* (lane 1); *atzB* (lane 2); *atzC* (lane 3); *atzD* (lane 4); *atzE* (lane 5); and *atzF* (lane 6). X molecular mass (MM) markers (Roche, Basel). Negative control (C).

Discussion

Strain MHP41 was identified as a species of the genus *Pseudomonas*, based on the analysis of phenotypic and biochemical characteristics. Because of their similar phenotypic characteristics and their active simazine degradation

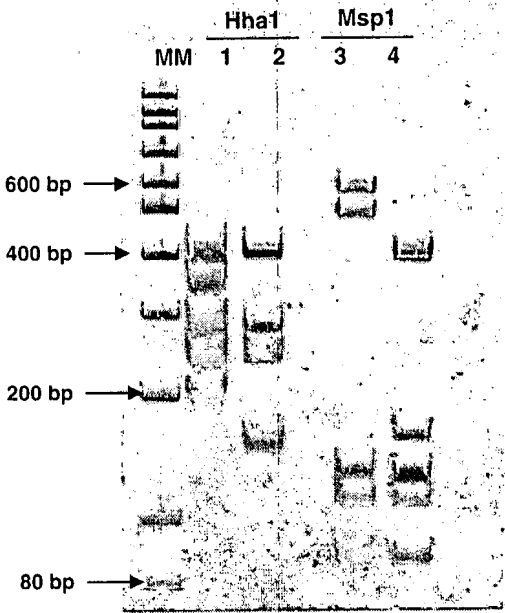


Fig. 3. ARDRA patterns of *Pseudomonas* sp. MHP41 and *Pseudomonas* sp. ADP. 16S rRNA gene PCR-amplification products of *Pseudomonas* sp. ADP (lines 1 and 3) and *Pseudomonas* sp. MHP41 (lines 2 and 4) were digested with HhaI and MspI. One hundred base pair molecular mass (MM) markers (Fermentas, ON) are shown on the left side.

Table 1. Compounds used as carbon sources for metabolism (respiration) of *Pseudomonas* sp. MHP41 in Biolog microplates

Carbon sources			
<i>Carboxylic acids</i>	<i>Amino acids</i>	<i>Polymers</i>	<i>Amides and Amines</i>
Acetic acid	D-Alanine	Dextrin	L-Alaninamide
cis-Aconitic acid	L-Alanine	Glycogen	Phenylethylamine
Citric acid	L-Alanyl-glycine	Tween 40	Putrescine
Formic acid	L-Asparagine	Tween 80	2-Aminoethanol
D-Gluconic acid	L-Aspartic acid		
α-Hydroxybutyric acid	L-Glutamic acid	<i>Carbohydrates</i>	
β-Hydroxybutyric acid	Glycyl-L-aspartic acid	N-Acetyl-D-glucosamine	
Itaconic acid	Glycyl-L-glutamic acid	D-Fructose	
α-Keto butyric acid	Hydroxy-L-proline	α-D-Glucose	
α-Keto glutaric acid	L-Leucine		
α-Keto valeric acid	L-Ornithine	<i>Esters</i>	
D,L-Lactic acid	L-Proline	Pyruvic acid methyl ester	
Propionic acid	L-Serine	Succinic acid mono-methyl-ester	
Quinic acid	L-Threonine		
Sebacic acid	D,L-Carnitine		
Succinic acid	γ-Amino butyric acid		
Bromosuccinic acid	Urocanic acid		

Table 2. Sequence similarities of *Pseudomonas nitroreducens* MHP41 16S rRNA gene (EBI Accession number: AM922106) to the respective sequences from other *Pseudomonas* species

<i>P. nitroreducens</i> MHP41 (CCUG 50378)			
		16S rRNA gene	
<i>P. multiresinovorans</i>	(lpA-1 = CCUG 51655-T)	X96787	99.8
<i>P. nitroreducens</i>	(DSM 14399 = CCUG 12538-T)	AM088474	99.7
<i>Pseudomonas</i> sp.	(ADP = CCUG 53068)	AM088478	98.9
<i>P. citronellolis</i>	(DSM 50332 = CCUG 17933-T)	Z76659	98.4
<i>P. alcaligenes</i>	(LMG 1224 = CCUG 1425-T)	Z76651	97.3
<i>P. aeruginosa</i>	(ATCC 27853 = CCUG 17619)	AY268175	96.9
<i>P. pseudoalcaligenes</i>	(ATCC 17440 = CCUG 51525-T)	Z76666	96.7

Pseudomonas species strain designations are in parentheses. Accession numbers and similarity values are indicated for 16S rRNA genes. 16S rRNA gene sequence similarities were calculated from alignments of nearly complete gene sequences: 1462 nucleotide positions, corresponding to *Escherichia coli* 16S rRNA gene sequence positions 28–1490.

activities, strains MHP41 and *Pseudomonas* sp. ADP were analysed by ARDRA profiling and were observed to exhibit significantly different 16S rRNA gene sequence restriction patterns (Fig. 3). Subsequently, on the basis of 16S rRNA gene sequence data, strain MHP41 was confirmed as a species of the genus *Pseudomonas*. The comparative 16S rRNA gene sequence analyses further suggested that strain MHP41 is closely related to the species *P. nitroreducens* and *P. multiresinovorans* and distinct from *P. citronellolis* as well as from strain *Pseudomonas* sp. ADP. These data indicated that strain MHP41 is not a strain of the same species as *Pseudomonas* sp. ADP and that a new simazine-degrading *Pseudomonas* species had been isolated. The levels of similarities observed between the 16S rRNA gene sequences of strain MHP41 and *P. nitroreducens* and *P. multiresinovorans* suggest that strain MHP41 could be a strain of one of those species. However, it is accepted that 16S rRNA gene sequence comparisons may indicate species-level identifications with probability but are not considered to be definitive. Thus, strain MHP41 should be considered to be a *Pseudomonas* sp., with a close phylogenetic relationship with *P. nitroreducens*.

The recognition that soil organisms can influence ecosystem processes has led to increased soil environmental studies (Ralebitso et al., 2002). Microorganisms are reported to be the primary players responsible for the removal of *s*-triazine herbicides in soil (Ralebitso et al., 2002; Santiago-Mora et al., 2005). Isolation of *s*-triazine-degrading bacteria has been reported in Europe, North America and central Asia. In this study, we have described the isolation and characterization of a novel simazine-degrading bacterium, *Pseudomonas* sp. strain MHP41, which was isolated from simazine-treated agricultural soil in Chile. This is the first report of an

s-triazine-degrading bacterial strain isolated in South America. This study further establishes the world-wide distribution of *s*-triazine-degrading bacteria.

A diverse range of microbial taxa capable of aerobic degradation of xenobiotic compounds have been reported (Seeger et al., 1999, 2001; Wackett, 2003; Cámara et al., 2004; Chain et al., 2006; Agulló et al., 2007; Martínez et al., 2007). Among them, *Pseudomonas* species are significant catabolic 'players', due to their capabilities of degrading a variety of different contaminants (Díaz, 2004). The genus *Pseudomonas* is present in ecologically diverse niches, playing important roles in the biogeochemical cycles. Bacteria belonging to this genus are well known for their metabolic versatility and their active function in aerobic degradation. They play key roles in biodegradation processes of xenobiotic compounds, e.g., polychlorobiphenyls, trinitrotoluene, aromatic hydrocarbons, azo dyes and pesticides (Zylstra et al., 1988; Göbel et al., 2002; Barreiros et al., 2003; Teitzel & Parsek, 2003; Wackett, 2003). Strains of *Pseudomonas* able to degrade atrazine and to use it as the sole nitrogen source have been isolated previously (Cook & Hütter, 1984; Mandelbaum et al., 1995).

Interestingly, this study shows that the novel *Pseudomonas* sp. MHP41 attains a high biomass using simazine as the sole nitrogen source (turbidity_{600 nm} of 1.03). In contrast, in similar media, other *s*-triazine-degrading bacteria attain lower biomass. Gram-negative strains M91-3 and CDB21 and Gram-positive *Arthrobacter* strain TC1 attain a turbidity_{600 nm} < 0.6, whereas *Arthrobacter* strain AD1 attains a turbidity_{600 nm} < 0.9 (Radosevich et al., 1995; Strong et al., 2002; Cai et al., 2003; Iwasaki et al., 2007). Although *Pseudomonas* sp. MHP41, with simazine as the sole N source, showed a relatively high growth rate ($\mu = 0.10 \text{ h}^{-1}$), *Pseudomonas* sp. ADP, using atrazine as an N source, showed faster growth ($\mu = 0.22 \text{ h}^{-1}$) (Neumann et al., 2004). Additionally, resting cells of strain MHP41 degrade more than 80% of the simazine within 60 min. Other bacterial strains demonstrated lower simazine degradation rates (Topp et al., 2000b; Iwasaki et al., 2007).

In this study, it was shown that *Pseudomonas* sp. MHP41 possesses the *s*-triazine catabolic *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes. Relatively few *s*-triazine-degrading strains have been described that possess the six genes encoding the enzymes for mineralization of these herbicides (Mandelbaum et al., 1995; Rousseaux et al., 2001; Devers et al., 2007). These *atz* genes are highly conserved among a taxonomic range of bacteria. A relationship between the presence of all the *atz* genes and the mineralization abilities of bacterial strains has been described previously (Rousseaux et al., 2001; Santiago-Mora et al., 2005; Devers et al., 2007). *Pseudomonas* sp. ADP possesses the complete atrazine degradation pathway encoded by the *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes (Mandelbaum et al., 1995) and is able to mineralize atrazine.

Some strains able to degrade atrazine and simazine possess only the 'upper' degradative pathway encoded by the *atzABC* genes, such as *Pseudoaminobacter* sp. C147 and *Chelatobacter heintzii* Cit1 (Topp *et al.*, 2000b; Rousseaux *et al.*, 2001). The *atzB* and *atzC* genes have been described in *Arthrobacter crystallopoietes* Cit2 and *Arthrobacter aurescens* TC1 (Rousseaux *et al.*, 2001; Strong *et al.*, 2002). *Stenotrophomonas maltophilia* Lous3-4 and *Aminobacter aminovorans* Ep2-1c possess only *atzA* and *atzB*, respectively (Rousseaux *et al.*, 2001). On the other hand, in *Ralstonia pickettii* strain D and *Agrobacterium radiobacter* J14a, only the 'lower' degradative pathway (*atzDEF*) has been detected (Cheng *et al.*, 2005). As diverse *s*-triazine degrading microorganisms have several catabolic *atz* gene combinations, the *atz* genes appear to be the result of a recent evolution.

In this work, we have reported the isolation and characterization of *Pseudomonas* sp. MHP41 from Chilean soils. This novel *s*-triazine-degrading bacterium efficiently degrades simazine, is capable of growing fast using simazine as the sole nitrogen source and yielding a high biomass. *Pseudomonas* sp. MHP41 possesses all *atz* genes of the upper and lower catabolic pathways for simazine degradation and is an interesting bacterium for studies on the bioremediation of *s*-triazine-contaminated soils.

Acknowledgements

The authors thank Larry Wackett for providing *Pseudomonas* sp. strain ADP, and Colin Campbell, Cecilia Flores and Pola Miralles for support. The authors are grateful for support from the Millennium Nucleus of Microbial Ecology and Environmental Microbiology and Biotechnology grant P04/007-F, USM 130522, INCO ICA4-CT-2002-10011 grant (European Union), Macaulay Development Trust and the Soil Health Initiative of the Macaulay Institute and Aberdeen University. M.H., P.V. and V.M. gratefully acknowledge CONICYT and MECESUP-UCV-0206 fellowships.

References

- Agulló L, Cámara B, Martínez P, Latorre-Reyes V & Seeger M (2007) Response to (chloro)biphenyls of the PCB-degrader *Burkholderia xenovorans* LB400 involves stress proteins induced also by heat shock and oxidative stress. *FEMS Microbiol Lett* 267: 167–175.
- Barreiros L, Nogales B, Manaia CM, Ferreira ACS, Pieper DH, Reis MA & Nunes OC (2003) A novel pathway for mineralization of the thiocarbamate herbicide molinate by a defined bacterial mixed culture. *Environ Microbiol* 5: 944–953.
- Cai B, Han Y, Liu B, Ren Y & Jiang S (2003) Isolation and characterization of an atrazine-degrading bacterium from industrial wastewater in China. *Lett Appl Microbiol* 36: 272–276.
- Cámara B, Herrera C, González M, Couve E, Hofer B & Seeger M (2004) From PCBs to highly toxic metabolites by the biphenyl pathway. *Environ Microbiol* 6: 842–850.
- Chain PS, Denev VJ, Konstantinidis KT *et al.* (2006) *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. *Proc Natl Acad Sci USA* 103: 15280–15287.
- Cheng G, Shapir N, Sadowsky MJ & Wackett LP (2005) Allophanate hydrolase, not urease, functions in bacterial cyanuric acid metabolism. *Appl Environ Microbiol* 71: 4437–4445.
- Cook AM & Hütter R (1984) Deethylsimazine: bacterial dechlorination, deamination, and complete degradation. *J Agric Food Chem* 32: 581–585.
- De Souza ML, Newcombe D, Alvey S, Crowley DE, Hay A, Sadowsky MJ & Wackett LP (1998a) Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. *Appl Environ Microbiol* 64: 178–184.
- De Souza ML, Seffernick J, Martinez B, Sadowsky MJ & Wackett LP (1998b) The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J Bacteriol* 180: 1951–1954.
- Devers M, Soulas G & Martin-Laurent F (2004) Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. *J Microbiol Methods* 56: 3–15.
- Devers M, Azhari NE, Kolic N-U & Martin-Laurent F (2007) Detection and organization of atrazine-degrading genetic potential of seventeen bacterial isolates belonging to divergent taxa indicate a recent common origin of their catabolic functions. *FEMS Microbiol Lett* 273: 78–86.
- Díaz E (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *Int Microbiol* 7: 173–180.
- Dinamarca MA, Cereceda-Balic F, Fadic X & Seeger M (2007) Analysis of *s*-triazine-degrading microbial communities in soils using most-probable-number enumeration and tetrazolium-salt detection. *Int Microbiol* 10: 209–215.
- Foth H (1999) Ecotoxicology. *Toxicology* (Marquardt H, Schäfer SG, Clellan RMc & Welsch F, eds), p. 1081. Academic Press, San Diego.
- García-González V, Govantes F, Shaw LJ, Burns RG & Santero E (2003) Nitrogen control of atrazine utilization in *Pseudomonas* sp. strain ADP. *Appl Environ Microbiol* 69: 6987–6993.
- Göbel M, Kassel-Cati K, Schmidt E & Reineke W (2002) Degradation of aromatics and chloroaromatics by *Pseudomonas* sp. strain B13: cloning, characterization, and analysis of sequences encoding 3-oxoadipate:succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. *J Bacteriol* 184: 216–223.
- Gunasekara AS, Troiano J, Goh KS & Tjeerdema RS (2007) Chemistry and fate of simazine. *Rev Environ Contam Toxicol* 189: 1–23.
- Hauben L, Vauterin L, Swings J & Moore ER (1997) Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int J Syst Bacteriol* 47: 328–335.

- Iwasaki A, Takagi K, Yoshioka Y, Fujii K, Kojima Y & Harada N (2007) Isolation and characterization of a novel simazine-degrading beta-proteobacterium and detection of genes encoding *s*-triazine-degrading enzymes. *Pest Manage Sci* 63: 261–268.
- Kulikova T, Akhtar R, Aldebert P et al. (2007) EMBL nucleotide sequence database in 2006. *Nucleic Acids Res* 35: D16–D20.
- Mandelbaum RT, Allan DL & Wackett LP (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. *Appl Environ Microbiol* 61: 1451–1457.
- Martínez B, Tomkins J, Wackett LP, Wing R & Sadowsky MJ (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J Bacteriol* 183: 5684–5697.
- Martínez P, Agulló L, Hernández M & Seeger M (2007) Chlorobenzoate inhibits growth and induces stress proteins in the PCB-degrading bacterium *Burkholderia xenovorans* LB400. *Arch Microbiol* 188: 289–297.
- Neumann G, Teras R, Monson L, Kivisaar M, Schauer F & Heipieper HJ (2004) Simultaneous degradation of atrazine and phenol by *Pseudomonas* sp. strain ADP: effects of toxicity and adaptation. *Appl Environ Microbiol* 70: 1907–1912.
- Pearson WR & Lipman DJ (1988) Improved tools for biological sequence comparison. *Proc Nat Acad Sci USA* 85: 2444–2448.
- Radosevich M, Traina SJ, Hao YL & Tuovinen OH (1995) Degradation and mineralization of atrazine by a soil bacterial isolate. *Appl Environ Microbiol* 61: 297–302.
- Ralebitso TK, Senior E & van Versveld HW (2002) Microbial aspects of atrazine degradation in natural environments. *Biodegradation* 13: 11–19.
- Rousseaux S, Hartmann A & Soulas G (2001) Isolation and characterisation of new gram-negative and gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiol Ecol* 36: 211–222.
- Santiago-Mora R, Martin-Laurent F, de Prado R & Franco AR (2005) Degradation of simazine by microorganisms isolated from soils of Spanish olive fields. *Pest Manage Sci* 61: 917–921.
- Seeger M, Zielinski M, Timmis KN & Hofer B (1999) Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the bph-encoded catabolic pathway of *Burkholderia* sp. strain LB400. *Appl Environ Microbiol* 65: 3614–3621.
- Seeger M, Cámara B & Hofer B (2001) Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase. *J Bacteriol* 183: 3548–3555.
- Strong LC, Rosendahl C, Johnson G, Sadowsky MJ & Wackett LP (2002) *Arthrobacter aureus* TC1 metabolizes diverse *s*-triazine ring compounds. *Appl Environ Microbiol* 68: 5973–5980.
- Tappe W, Groeneweg J & Jantsch B (2002) Diffuse atrazine pollution in German aquifers. *Biodegradation* 13: 3–10.
- Teitzel GM & Parsek MR (2003) Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 69: 2313–2320.
- Topp E, Mulbry WM, Zhu H, Nour SM & Cuppels D (2000a) Characterization of *s*-triazine herbicide metabolism by a *Nocardioide* sp. isolated from agricultural soils. *Appl Environ Microbiol* 66: 3134–3141.
- Topp E, Zhu H, Nour SM, Houot S, Lewis M & Cuppels D (2000b) Characterization of an atrazine-degrading *Pseudaminobacter* sp. isolated from Canadian and French agricultural soils. *Appl Environ Microbiol* 66: 2773–2782.
- Wackett LP (2003) *Pseudomonas putida* – a versatile biocatalyst. *Nat Biotechnol* 21: 136–138.
- Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173: 697–703.
- Zylstra GJ, McCombie WR, Gibson DT & Finette BA (1988) Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. *Appl Environ Microbiol* 54: 1498–1503.

ORIGINAL ARTICLE

Simazine treatment history determines a significant herbicide degradation potential in soils that is not improved by bioaugmentation with *Pseudomonas* sp. ADP

A.C. Morán¹, A. Müller², M. Manzano¹ and B. González¹

¹ Laboratorio de Microbiología, Departamento de Genética Molecular y Microbiología, and Center for Advanced Studies in Ecology and Biodiversity, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

² Institut für Mikrobiologie, Universität Stuttgart, Stuttgart, Germany

Keywords

bioremediation, *Pseudomonas* sp., simazine, terminal restriction fragment length polymorphism.

Correspondence

Bernardo González, Laboratorio de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. E-mail: bgonzalez@bio.puc.cl

2005/1010: received 6 September 2005, revised and accepted 9 December 2005

doi:10.1111/j.1365-2672.2006.02990.x

Abstract

Aims: To study biological removal of the herbicide simazine in soils with different history of herbicide treatment and to test bioaugmentation with a simazine-degrading bacterial strain.

Methods and Results: Simazine removal was studied in microcosms prepared with soils that had been differentially exposed to this herbicide. Simazine removal was much higher in previously exposed soils than in unexposed ones. Terminal restriction fragment length polymorphism analysis and multivariate analysis showed that soils previously exposed to simazine contained bacterial communities that were significantly impacted by simazine but also had an increased resilience. The biodegradation potential was also related to the presence of high levels of the *atz*-like gene sequences involved in simazine degradation. Bioaugmentation with *Pseudomonas* sp. ADP resulted in an increased initial rate of simazine removal, but this strain scarcely survived. After 28 days, residual simazine removals were the same in bioaugmented and not bioaugmented microcosms.

Conclusions: In soils with a history of simazine treatment bacterial communities were able to overcome subsequent impacts with the herbicide. The success of bioaugmentation was limited by the low survival of the introduced strain.

Significance and Impact of the Study: Conclusions from this work provided insights on simazine biodegradation potential of soils and the convenience of bioaugmentation.

Introduction

Atrazine and simazine are *s*-triazine herbicides used worldwide in agriculture, since the 1950s. These herbicides interact with the quinone-binding protein in photosystem II, thus preventing the electron transport in susceptible weeds (Wackett *et al.* 2002). Atrazine half-life in soils ranges between 4 and 57 weeks (Best and Weber 1974). This relatively long time of persistence and its possible threats for health lead to extensive investigations on atrazine degradation in contaminated soils, which is mostly performed by micro-organisms. Several atrazine-

degrading microbial associations and monocultures able to utilize atrazine as nitrogen and/or carbon source have been isolated from treated soils (Behki *et al.* 1993; Mandelbaum *et al.* 1995; Struthers *et al.* 1998; Topp *et al.* 2000; Rousseaux *et al.* 2001, 2002; Ghosh and Philip 2004; Singh *et al.* 2004). Among them, *Pseudomonas* sp. ADP has become a useful model to elucidate the aerobic catabolic pathway, encoded by the *atzA*, *-B*, *-C*, *-D*, *-E* and *-F* genes (Ralebitso *et al.* 2002; Wackett *et al.* 2002). These genes are located on the 109-kb, self-transmissible, broad host-range plasmid pADP-1 (de Souza *et al.* 1998b; Martínez *et al.* 2001). Although several works about

atrazine biodegradation in soil and the effect of inoculation with atrazine-degrading strains have been reported, only recently literature refers to simazine, a very close but less soluble analogue of atrazine which has also been used worldwide (Strandberg and Scott-Fordsmand 2002; Alister *et al.* 2005; Barra-Caracciolo *et al.* 2005; Sánchez *et al.* 2005; Santiago-Mora *et al.* 2005). Therefore, in this work we study the microbial degradation of simazine in soils with different biodegradation potentials and the effect of bioaugmentation with the *Pseudomonas* sp. ADP strain.

Materials and methods

Bacterial strain and growth conditions

Pseudomonas sp. ADP (p-ADP1) was cultured in a minimal medium (Kröckel and Focht 1987), without ammonium sulfate and supplemented with 10 g l^{-1} of trisodium citrate dihydrate as carbon source. For liquid and solid cultures, 0.1 and 0.5 g l^{-1} of simazine as nitrogen source were added, respectively.

Soil sampling

Soil samples were taken from a 28-year-old avocado (*Persea americana*) plantation, located in central Chile that had been treated for the last 10 years with commercial simazine to control annual weeds. The samplings included soils treated with herbicide according to standard farming practices, and a control soil from a zone in the limit of the avocado plantation which had no agricultural use, nor herbicide exposure history. Samples of about 1 kg were taken from the first 20 cm of depth, at a distance of 0.5 m from 10 randomly selected avocado trees, and then pooled and sieved through a 5-mm stainless steel sieve, air dried and stored in polyethylene bags at 4°C . The agricultural soil and the control soil were sandy silty loamy and silty loamy, with pHs of 6.55 and 6.42, respectively. Carbon and nitrogen content were 2.6% and 0.20% for the agricultural soil and 1.3% and 0.10% for the control soil, respectively (E. Moore, personal communication). Samples of 10- and 11-year treated soils were taken in December 2002, 6 months after simazine field application (soil A), and in June 2003, 1 month after treatment (soil B), respectively. The first had no detectable simazine and the latter contained about 2 ppm simazine at the time of sampling (F. Cereceda and M. Seeger, personal communication).

Soil microcosm experiments

Previous to microcosm set-up, the stored soils were sieved through a 2-mm sieve and air dried. For the first

series of microcosms, control soil and the agricultural soil A were used. Both soils had been sampled 3 weeks before the start of the experiment. Microcosms were prepared in 50-ml glass beakers with 5 g of soil. Simazine (Gesatop, 90% purity; Syngenta, Santiago, Chile) was added by pipetting 3.5 ml of agitated simazine aqueous suspensions containing 7.14 or 57.1 mg l^{-1} to reach final concentrations of 5 and 40 ppm. The same amount of distilled water was added to 0 ppm microcosms. Beakers were covered with polyethylene foil and incubated at $22 \pm 5^\circ\text{C}$. In order to assess the extent of biological degradation, poisoned controls of the 40 ppm microcosms were prepared by adding 1200 ppm of HgCl_2 (Stroetmann *et al.* 1994). Sterile distilled water was added twice a week to keep the moisture of soil. At days 0 and 60, two replicate samples for each condition were collected for DNA analysis and three replicates for residual simazine determinations. Each sample consisted of a complete microcosm. For a second series of experiments, agricultural soil A (amended with 0 or 40 ppm simazine) or B (amended with 40 ppm simazine) were used to prepare microcosms as described above. This experiment started 3 weeks after the sampling of soil B. For microcosms testing bioaugmentation, *Pseudomonas* sp. ADP was cultured in the conditions described above, for 72 h. Cells from a 150-ml culture were harvested by centrifugation at $10\,000 \text{ g}$ for 5 min, washed once with $\text{NaCl } 8.5 \text{ g l}^{-1}$, and then resuspended in 10 ml of $\text{NaCl } 8.5 \text{ g l}^{-1}$. Colony-forming units (CFU) were counted on R2A agar plates. A volume of $100 \mu\text{l}$ of the 10 ml cell concentrate contained 5×10^8 CFU, the amount required to inoculate 10^8 cells per gram of soil in microcosms prepared with soil A. Two microcosms for DNA analysis and three for simazine determinations were collected after 0, 14 and 28 days.

Simazine concentration measurement

For extracting simazine from soil samples, 15 ml of a 3 : 1 methanol/water solution were added to each microcosm. The mixture was transferred to a Teflon 50-ml bottle, agitated in a shaker at $200 \text{ cycles min}^{-1}$ for 30 min, left to stand overnight, and then agitated again for 30 min. Soil particles were allowed to settle for approx. 15 min and 2 ml of extract were clarified by centrifugation at $20\,000 \text{ g}$, for 5 min. Simazine concentration was measured in these extracts, properly diluted in water, using the Rapid Assay® Kit (Strategic Diagnostics Inc., Newark, NJ, USA).

Soil DNA isolation

The extraction of DNA from each sample was carried out using the 'Ultra Clean Soil DNA Kit' (MoBio Laborator-

ies, Inc. Solana Beach, CA, USA). After the lysis step, performed following the manufacturer's protocol, a cetyl-trimethylammonium bromide (CTAB) purification step was included, which consisted of the addition of 5 mol l⁻¹ NaCl (0.7 mol l⁻¹ final concentration), and then CTAB (10% CTAB in 0.7 mol l⁻¹ NaCl) to a final concentration of 1% CTAB. The sample was incubated at 65°C for 20 min, extracted with one volume of chloroform : iso-amyl alcohol (24 : 1) and centrifuged at 6000 g for 10 min. This protocol yielded DNA concentrations ranging from 0.03 to 0.06 µg µl⁻¹. Extracted DNA was visualized in 0.8% agarose gels.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

Soil community DNA was used as template for PCR with the primers 8F: 5'-AGA GTT TGA TCC TGG CTC AG-3', labelled with the fluorochrome NED, and 1392R: 5'-ACG GGC GGT GTG TAC-3', designed for eubacterial 16S rRNA gene sequences (Amann et al. 1995). For each DNA sample, three 50-µl reactions were performed, containing 5 µl of 10x PCR buffer (200 mmol l⁻¹ Tris-HCl pH 8, 500 mmol l⁻¹ KCl), 3 mmol l⁻¹ MgCl₂, 0.2 µmol l⁻¹ of each primer, 0.2 mmol l⁻¹ dNTP, 0.2 mg ml⁻¹ bovine seroalbumine, c. 10–50 ng of soil DNA and 1 U of *Taq* polymerase. Reaction conditions were as follows: 94°C for 5 min, 25 cycles at 94°C for 45 s, 57°C for 45 s, and 72°C for 2 min, and finally, 7 min at 72°C. The pooled PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and digested with *MspI* or *HhaI* (Promega). After desalting, the DNA fragments were separated by capillary electrophoresis using a Perkin-Elmer (Foster City, CA, USA) ABI Prism 310 Sequence Detection System. The fragment sizes were estimated using as reference the internal standard ROX 1000.

T-RFLP data handling and multivariate analyses

Raw data sets consisted of peaks reflecting the size of terminal restriction fragments (T-RFs), measured in base pairs, and the area and height of each peak measured in fluorescence units. T-RFs from 50 to 600 bp were included in the analysis except those representing <0.5% of the total area. Data were standardized by calculating the area of each peak as a percentage of the total area. The T-RF relative areas were analysed with the multivariate statistical software Primer 5 (Primer-E Ltd, Plymouth, UK). Principal component analyses were used to group data from T-RFLP profiles. Similarity matrices were calculated using the Bray-Curtis coefficient (Rees et al. 2004). With those matrices, analysis of similarity

(ANOSIM), a multivariate randomization procedure analogous to ANOVA, was carried out to examine the statistical significance of grouping. The output statistics, the global *R* takes a value of 0 if there is no separation of the community structure due to the factor analysed, and 1 if total separation takes place (Clarke 1993). Values higher than 0.5 account for high differences, whereas between 0.25 and 0.5 differences are considered as moderate. Cluster analysis based on similarity matrices were performed using the group average method. The T-RFLP Phylogenetic Assignment Tool (Kent et al. 2003) was used to construct taxonomic profiles, by assigning the peaks obtained with *MspI* and *HhaI*-T-RFLPs to bacterial phyla. Within Proteobacteria and Bacteroidetes phyla, bacterial classes were considered. About 99% of *MspI* peak areas matched with known 16S rRNA gene sequences whereas for *HhaI* peaks the average area assignment percentage was 90%. Then, *MspI*-T-RFLP peaks were used to calculate the relative abundance percentages of each group by assigning a fraction of the area of the peak to each possible bacterial phylum or class matching a particular fragment length.

Detection of catabolic genes

The *atzA* and *atzC* gene sequences were detected in soil samples by PCR amplification with the primers described by de Souza et al. (1998a). Each PCR was performed in a total volume of 20 µl, containing 2 µl of 10x PCR buffer (200 mmol l⁻¹ Tris-HCl pH 8, 500 mmol l⁻¹ KCl), 3 mmol l⁻¹ MgCl₂, 0.2 µmol l⁻¹ of each primer, 0.2 mmol l⁻¹ dNTP, c. 10–50 ng of soil DNA and 1 U of *Taq* polymerase. Reaction conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 30 s, and finally, 7 min at 72°C. The presence of the *trzN* gene sequence was investigated by PCR using the primers and amplification conditions set up by Mulbry et al. (2002). PCR products for *atzA* and *atzC* sequences obtained from community DNA were cloned into the pCR2.1 vector (TOPO TA Cloning System; Invitrogen, Carlsbad, CA, USA) and sequenced using a PRISM sequencing kit (Perkin-Elmer) on a 373 stretch automated sequencer (Applied Biosystems, Foster City, CA, USA).

For competitive PCR of *atzA* gene sequences, a competitor DNA was constructed by cloning a 527-bp PCR *atzA* product from *Pseudomonas* sp. ADP (pADP-1) into the pCR2.1 vector and deleting an internal fragment with the enzyme *BsmI*. The resulting vector produced a 357-bp PCR product with the *atzA* primers. The concentration of the vector used as competitor was measured by UV spectrophotometry. Amplification conditions for competitive PCR were as described above, but with 25 cycles. PCR products were visualized in 2% agarose gels.

Results

Simazine treatment history determines simazine biodegradation potential in soils

Simazine biodegradation potential in soil was assessed by preparing microcosms with both, previously treated and nontreated soils, amended with 5 ppm simazine, the concentration reported for soils after a regular simazine field application, or 40 ppm, an eightfold higher concentration. Residual simazine percentages, shown in Fig. 1, illustrate the effect of the treatment history on the ability to degrade simazine. It can be observed that in both soils exposed to 5 ppm simazine, most of the added herbicide was removed after 60 days. In contrast, in soils amended with 40 ppm simazine, clear differences in the degradation potential of the soil previously treated with simazine (soil A) and the untreated soils were observed. The removal levels obtained with the untreated control soil were similar to that observed in soil microcosms treated with HgCl_2 , a common-use germicide (Strandberg and Scott-Fordsmand 2002). The treatment with HgCl_2 decreased to one-tenth the number of soil micro-organisms, as judged by the CFU ml^{-1} on R2A agar plates. These results clearly indicate that microbial removal of simazine in these soil microcosms is affected by simazine level and the previous simazine treatment of soils.

Bacterial communities were assessed by T-RFLP in these microcosms and ANOSIM of *MspI*-T-RFLP data was used to evaluate the effect of experimental variables on the structure of bacterial communities. The type of soil (10-year treated vs control) affected bacterial communities more

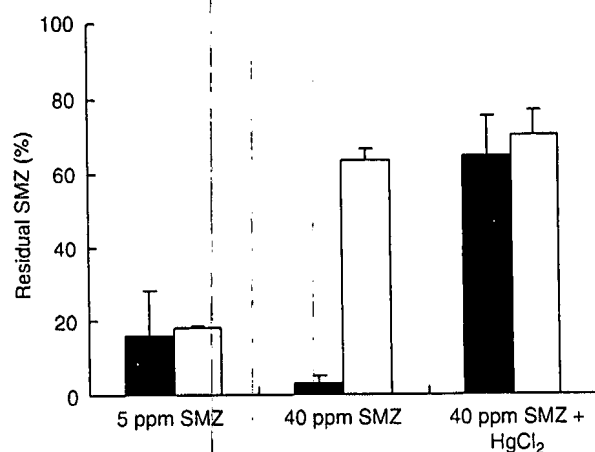


Figure 1 Residual simazine measured in soil microcosms prepared with 10-year treated soil A (■) or control soil (□) after 60 days of incubation. SMZ = simazine. Error bars represent standard deviations for measurements of three replicate samples.

than the addition of simazine or the soil incubation, with a global $R = 0.815$ ($P = 0.001$) for the first factor, and low and not significant values of R ($P > 0.05$), for the other factors. To assess the effect of the other factors, the data for different soils were considered separately. Principal component analysis (PCA) of *MspI*-T-RFLP data revealed that the main ordinating component (PC1) in the case of 10-year treated soil was related with the incubation time (Fig. 2a). This fact was also supported by ANOSIM, which yielded an R -value of 0.76 ($P = 0.036$) for the incubation time whereas the factor simazine gave a low and not significant ($P > 0.05$) value of R . Conversely, for control soils (Fig. 2b), PC1 was related with simazine amendment and ANOSIM showed that this factor, with an $R = 0.813$ ($P = 0.029$), affected bacterial communities more than incubation time.

Exposure to simazine affects bacterial degradation potential for simazine in soils

In a second series of microcosms, we compared the kinetics of simazine removal in microcosms amended with

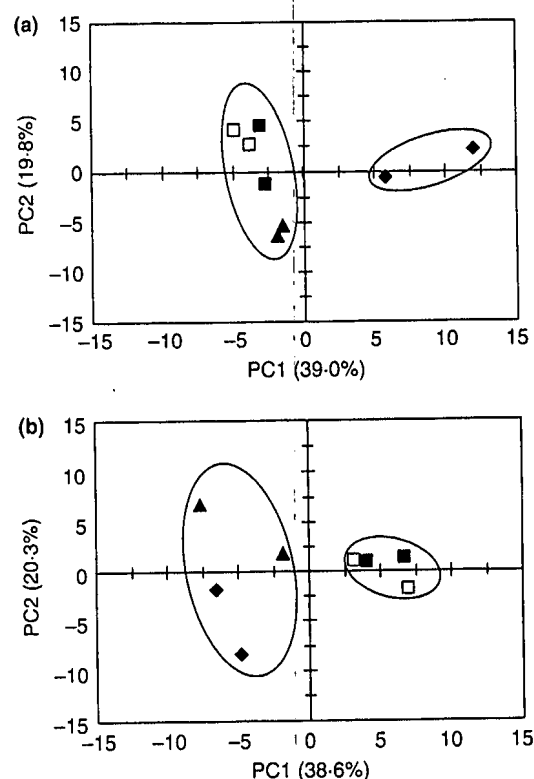


Figure 2 Principal component analysis (PCA) of *MspI*-T-RFLP profiles. (a) Ten-year treated soil A or (b) control soil microcosms at 0 (◆) or 60 days of incubation amended with 0 (▲), 5 (□) or 40 ppm simazine (■). The lines around data points indicate the groups formed in cluster analysis.

40 ppm simazine and prepared with the 10-year treated soil (hereafter soil A) and the same soil sampled 1 month after the eleventh yearly herbicide application in the field (soil B). The residual simazine concentration could be adjusted to an exponential kinetics ($[\text{simazine}] = e^{-kt}$), corresponding to a first-order reaction (Fig. 3). The constants (k) calculated for soils A and B were 0.06 and 0.23 day^{-1} , respectively, indicating that soil B has a more active degrading microbial population. For bioaugmented microcosms prepared with soil A, the highest initial rate of removal was observed, but data could not be fitted to an exponential decay. After 28 days of incubation, residual levels of simazine were similar for all microcosms.

ANOSIM of the *MspI*-T-RFLP data showed that the time of incubation was the main factor affecting the structure of bacterial communities ($R = 0.528$, $P = 0.001$). Therefore, data for 14 and 28 days were analysed separately. At 14 days of incubation (Fig. 4a), PCA showed that PC1 was related with simazine amendment whereas at 28 days (Fig. 4b) simazine amendment was related with PC2. Moreover, the R -value from ANOSIM considering the factor 'simazine amendment', was equal to 1 ($P = 0.036$) at 14 days and decreased to 0.813 ($P = 0.036$) after 28 days of incubation. As it can be observed in Fig. 4, data of inoculated microcosms did not differ from the non-inoculated microcosms. The 73-bp *MspI*-T-RFLP peak corresponding to *Pseudomonas* sp. ADP (pADP-1), which had about 16% of relative area just after inoculation, decreased to <1% after 14 days of incubation.

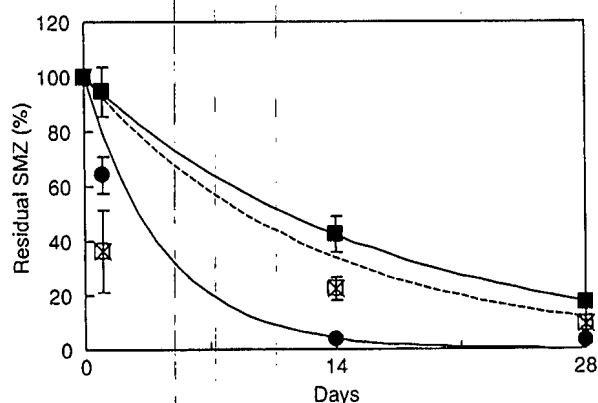


Figure 3 Time course for simazine degradation in microcosms prepared with soil A (■), soil A bioaugmented with 10^8 CFU of *Pseudomonas* sp. ADP (pADP-1) (□), and soil B (●), all amended with an initial concentration of 40 ppm simazine. The lines correspond to the equation of data fitted to an exponential decay, with R^2 values of 0.99 for the continuous lines, and 0.62 for the dotted line which correspond to the data of bioaugmented microcosms. SMZ = simazine. Error bars represent standard deviations for measurements of three replicate samples.

To identify bacterial groups whose relative abundance could be affected by simazine amendment, we compared bacterial groups from soil A microcosms amended with 0 and 40 ppm simazine after 14 days of incubation, the condition for which the highest effect of simazine amendment on the structure of bacterial communities was observed (Fig. 4a). The bacterial groups were assigned based on *MspI* and *HhaI*-T-RFLP data using the Phylogenetic Assignment Tool and results are shown in Fig. 5. The groups which increased with simazine amendment were β - and γ -Proteobacteria, as well as less abundant groups as Sphingobacteria and Chlorobi, whereas δ -Proteobacteria and Fusobacteria, clearly decreased after herbicide treatment. The pair-wise comparisons of T-RFLP profiles from 14-day samples showed that the clearest difference was in the 488-bp *MspI* signal, which increased from 4.5% to 12.3%. Among several matches found for this signal with the Phylogenetic Assignment Tool, some of the possible *HhaI* matches that were also increased with simazine treatment, corresponded to bacterial genera

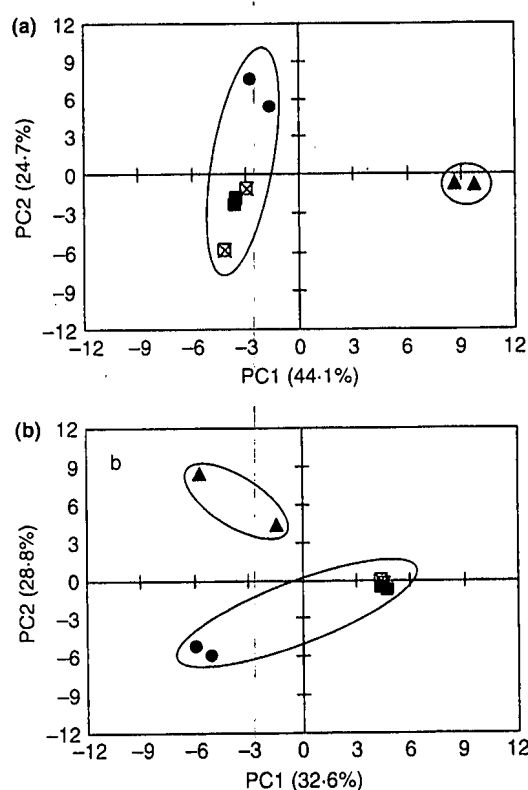


Figure 4 PCA of *MspI*-T-RFLP profiles of microcosms after (a) 14 or (b) 28 days of incubation, and prepared with soil A + 0 ppm SMZ (▲), soil A + 40 ppm SMZ (■), soil A + 40 ppm SMZ bioaugmented with 10^8 CFU of *Pseudomonas* sp. ADP (pADP-1) (□), and soil B + 40 ppm SMZ (●). The lines around data points indicate the groups formed in cluster analysis.

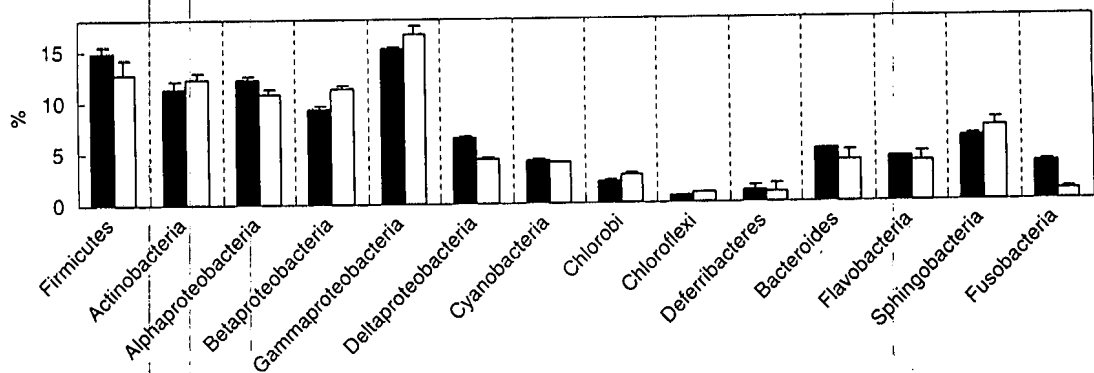


Figure 5 Relative abundance of bacterial groups detected by assignation of *MspI* and *HhaI*-T-RFLP signals obtained for soil A microcosms amended with 0 (■) and 40 ppm simazine (□) after 14 days of incubation. Error bars represent standard deviations for assignation of signals of two replicate samples.

Table 1 Copies of *atzA* gene sequences per gram of soil measured by competitive polymerase chain reaction*

	Soil A	Soil A + SMZ	Soil A + SMZ + ADP	Soil B + SMZ
0 days	<10 ⁶	<10 ⁶	7.7 × 10 ⁷	<10 ⁶
14 days	<10 ⁶	1.5 × 10 ⁷	1.1 × 10 ⁷	1.7 × 10 ⁷
28 days	<10 ⁶	<10 ⁶	<10 ⁶	<10 ⁶

*The detection limit was approx. 10⁶ copies of *atzA* gene sequences per gram of soil. Standard deviations were about 10%. SMZ, simazine; ADP, *Pseudomonas* sp. ADP.

of the γ -Proteobacteria class as *Pseudomonas*, *Acinetobacter* and *Acidovorax*.

Catabolic *atz* genes are detected in soils actively degrading simazine.

The presence in soil microbial DNA of the atrazine degradation gene sequences *atzA*, *atzC*, and *trzN*, was investigated by PCR. In microcosms prepared with soils A or B and amended with 40 ppm simazine, we detected PCR products of the expected size for the *atzA* and the *atzC* gene, only at time intervals in which simazine is being removed (data not shown). The relative amount of *atzA* gene sequences in the soil microbial population of the second series of microcosms was determined by competitive PCR detection (Table 1). Results indicated that the *atzA* gene sequence levels were clearly related to the removal rates reported in Fig. 3. Interestingly, in the first series of microcosms, no *atz* sequences were found in microcosms prepared with control soil. It should be mentioned that both genes encoding *s*-triazine dechlorinating activities, *atzA* and *trzN*, had been detected in treated soils shortly after field sampling, but only *atzA* gene was detected in soil microcosms. Six *atzA* and two *atzC*

cloned PCR products obtained from the soil microbial community DNA were sequenced. The sequencing revealed high similarity (>99%, in nucleotides) to the *atzA* and *atzC* genes from *Pseudomonas* sp. ADP.

Discussion

This study showed that history of simazine treatment increased the soil self-remediation potential, as it was reported for *s*-triazines and other pollutants (Shapir *et al.* 2000; Tuxen *et al.* 2002; Watanabe *et al.* 2002; Rhine *et al.* 2003). The usefulness of a soil with no agricultural history as the control soil may be questioned. However, the C/N ratio of these two soils, a parameter that could influence the degradation of a nitrogenated herbicide (García-González *et al.* 2003; Barra-Caracciolo *et al.* 2005), is the same. Then, the biodegradation potential that differentiated these soils seemed to be intrinsic to bacterial communities, which varied in structure, as was evidenced by ANOSIM of T-RFLP data, and the presence of catabolic genes related to *s*-triazine degradation. The biodegradation potential of soils is usually assessed by cultivation of bacteria able to degrade the target xenobiotic (Ahn *et al.* 1999; Runes *et al.* 2003). However, culture-dependent approaches are clearly biased (e.g. Marchesi and Weightman 2001) and in the case of simazine, cultivation is based on a very demanding selective condition, the ability to use this low solubility herbicide as sole nitrogen source (Topp *et al.* 2000; Rousseaux *et al.* 2001). Therefore, culture-independent approaches are more adequate to predict biodegradation potential. The presence in soil microbial DNA of *atzA* gene sequences, which encode the enzyme performing dechlorination of *s*-triazines, the first step in atrazine or simazine degradation (de Souza *et al.* 1996), is considered an indicator for the biodegradation potential of *s*-triazines (Shapir *et al.*

2000). In our study, the genes *atzA* and *atzC* were detected by PCR in conditions where high simazine removal rates were observed which agree with their use as biodegradation potential indicators. We additionally investigated the presence of *trzN* gene from *Nocardioide* sp. strain C190, a different gene encoding an enzyme able to dechlorinate atrazine and simazine (Mulbry et al. 2002; Piutti et al. 2003; Sajjaphan et al. 2004). However, we did not detect this gene in soil microcosms, although it had been detected shortly after soil field sampling. This fact would suggest that, in spite of the reliability of most conclusions arisen from microcosm studies, some bias occurs with respect to field simazine degradation. Moreover, in microcosms amended with the simazine concentration used in the field (5 ppm), catabolic genes could not be detected. A higher concentration of simazine (40 ppm) may have resulted in favouring *AtzA*-mediated simazine degradation over processes involving different *s*-triazine catabolic genes reported in the literature (Shao et al. 1995; Mulbry et al. 2002; Piutti et al. 2003). Highly conserved *atz* sequences have been frequently observed in herbicide-polluted environments (de Souza et al. 1998a; Martin-Laurent et al. 2003; Devers et al. 2004). The high conservation of *atz* sequences may be explained by the use of primers that are based on the *atz* sequences from *Pseudomonas* sp. ADP, but it has been also suggested that the strong sequence conservation in *atz* genes is a result of the recent evolution of these sequences due to the relatively recent use of these herbicides (Seffernick and Wackett 2001). On the other hand, the fact that *atz* genes are encoded in the broad host range, self-transmissible plasmid pADP-1, and therefore, allowing fast spread of these sequences into the bacterial community, may also explain the conservation of *atz* sequences detected in soils (de Souza et al. 1998b). In our microcosms exposed to high levels of simazine, a relatively higher efficiency of *atzA* gene products and the ability of *atz* genes harbouring plasmids to spread these genes in the bacterial community would be the factors determining its prevalence with respect to *trzN*.

Soil communities have been reported as highly resilient to acute perturbation, due to the high degree of functional redundancies (Griffiths et al. 2004; Pesaro et al. 2004; Girvan et al. 2005). Simazine degradation potential acquired by repeated herbicide treatment may also confer to bacterial communities a higher of resilience to the impact of the herbicide. This is well illustrated in Fig. 2b, where data from 5 and 40 ppm simazine amended control soil microcosms appeared clustered. Although the herbicide in the 5 ppm control soil microcosms had been removed to the same extent than in the 5 ppm 10-year treated soil microcosms (Fig. 1), bacterial communities remained as impact as those of the 40 ppm control soil

microcosms, where a high residual simazine was detected after 60 days of incubation. The functional redundancy of this control soil community seems not to be enough to overcome the impact of a recalcitrant compound as simazine, for which a low diversity of genes involved in its degradation has been reported (de Souza et al. 1998a), compared with the diversity of genes involved in degradation of less persistent herbicides as 2,4-D (Top et al. 1995; Itoh et al. 2002). In contrast, the 10-year treated soil microcosms showed a significant impact of simazine after 14 days of incubation (Fig. 4a), but this effect decreased after 28 days, where the factor 'simazine amendment' was no longer related to the PC1.

Simazine biodegradation potential in these soils might be related to the ability of β - and γ -Proteobacteria to proliferate during active herbicide removal, as judged by *MspI* and *HhaI*-T-RFLP assignation. Interestingly, bacterial genera that increased with simazine included bacterial strains already reported as atrazine degraders, such as *Pseudomonas* (Yanze-Kontchou and Gschwind 1994; Mandelbaum et al. 1995), *Acinetobacter* (Singh et al. 2004), and *Acidovorax*, strains formerly classified as *Pseudomonas* (Willems et al. 1992; Karns 1999; Seffernick et al. 2001).

Studies on the kinetics of simazine degradation have shown first-order decays for this herbicide (Strandberg and Scott-Fordsmand 2002; Alister et al. 2005). In our soil microcosms, soil B, containing a more recently exposed bacterial community to this herbicide, showed a higher first-order degradation constant than soil A. Bioaugmentation with the degrading strain *Pseudomonas* sp. ADP (pADP-1) did result in a highly increased initial rate of simazine removal, which correlated well with the high amount of *atzA* gene sequences detected at day 0 (Table 1). However, low survival of this strain was evident in the T-RFLP profiles obtained after 14 days of incubation (data not shown). Moreover, the *atzA* gene levels in bioaugmented soil microcosms rapidly decreased during incubation (Table 1), indicating that the inoculum did not survive or was unable to spread the plasmid pADP-1 to other bacteria. This low survival explains the lack of fit of residual simazine data to an exponential decay. In soils exposed to atrazine, success with inoculation of *Pseudomonas* sp. ADP has been reported but also a low survival of the inoculated cells was observed (Zhao et al. 2003). A repeated inoculation strategy (Newcombe and Crowley 1999), or the combination with biostimulation (Silva et al. 2004) has been applied to improve bioaugmentation performance in atrazine polluted soils. Our study indicates that to remove simazine in 1 or 2 months bioaugmentation is not required. However, bioaugmentation would be very helpful for efficient removal of most of the herbicide in shorter times. Then, the use of

bioaugmentation or alternatives such as the use of a killed recombinant producing AtzA described by Strong *et al.* (2000), would be advisable in the case of a herbicide spill to avoid herbicide leaching to the groundwater (Strandberg and Scott-Fordsmand 2002), which constitutes one of the main environmental concerns about using s-triazine herbicides.

Acknowledgements

This work was supported by FONDAP-FONDECYT (grant 1501-0001, program 7), and by ICA4-CT-2002-10011 (ACCESS) Contract of the European Union.

References

- Ahn, Y., Sanseverino, J. and Sayler, G.S. (1999) Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. *Biodegradation* 10, 149–157.
- Alistar, C., López, R. and Kogan, M. (2005) Simazine dynamics in a vineyard soil at Casablanca valley, Chile. *Pest Manag Sci* 61, 1083–1088.
- Amann, R., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbial Rev* 59, 143–169.
- Barra-Caracciolo, A.B., Grenni, P., Ciccoli, R., Di Landa, G. and Cremisini, C. (2005) Simazine biodegradation in soil: analysis of bacterial community structure by in situ hybridization. *Pest Manag Sci* 61, 863–869.
- Behki, R., Topp, E., Dick, W. and Germon, P. (1993) Metabolism of the herbicide atrazine by *Rhodococcus* strains. *Appl Environ Microbiol* 59, 1955–1959.
- Best, J.A. and Weber, J.B. (1974) Disappearance of s-triazines as affected by soil pH using a balance-sheet approach. *Weed Sci* 22, 364–373.
- Clarke, K.R. (1993) Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* 18, 117–143.
- Devers, M., Soulas, G. and Martin-Laurent, F. (2004) Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. *J Microbiol Methods* 56, 3–15.
- García-González, V., Govantes, F., Shaw, L.J., Burns, R.G. and Santero, E. (2003) Nitrogen control of atrazine utilization in *Pseudomonas* sp. strain ADP. *Appl Environ Microbiol* 69, 6987–6993.
- Ghosh, P.K. and Philip, L. (2004) Atrazine degradation in anaerobic environment by a mixed microbial consortium. *Water Res* 38, 2276–2283.
- Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I. and Glover, L.A. (2005) Bacterial diversity promotes community stability and functional resilience after perturbation. *Environ Microbiol* 7, 301–313.
- Griffiths, B.S., Kuan, H.L., Ritz, K., Glover, L.A., McCaig, A.E. and Fenwick, C. (2004) The relationship between microbial community structure and functional stability, tested experimentally in an upland pasture soil. *Microb Ecol* 47, 104–113.
- Itoh, K., Kanda, R., Sumita, Y., Kim, H., Kamagata, Y., Suyama, K., Yamamoto, H., Hausinger, R.P. *et al.* (2002) *tfdA*-like genes in 2,4-dichlorophenoxyacetic acid-degrading bacteria belonging to the Bradyrhizobium-Agromonas-Nitrobacter-Afipia cluster in alpha-Proteobacteria. *Appl Environ Microbiol* 68, 3449–3454.
- Karns, J.S. (1999) Gene sequence and properties of an s-triazine ring-cleavage enzyme from *Pseudomonas* sp. strain NRRLB-12227. *Appl Environ Microbiol* 65, 3512–3517.
- Kent, A.D., Smith, D.J., Benson, B.J. and Triplett, E.W. (2003) A web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Appl Environ Microbiol* 69, 6768–6776.
- Kröckel, L. and Focht, D. (1987) Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. *Appl Environ Microbiol* 53, 2470–2475.
- Mandelbaum, R.T., Allan, D.L. and Wackett, L.P. (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Appl Environ Microbiol* 61, 1451–1457.
- Marchesi, J.R. and Weightman, A.J. (2001) Comparing the dehalogenase gene pool in cultivated alpha-halocarboxylic acid-degrading bacteria with the environmental metagenome pool. *Appl Environ Microbiol* 69, 4375–4382.
- Martínez, B., Tomkins, J., Wackett, L.P., Wing, R. and Sadowsky, M.J. (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J Bacteriol* 183, 5684–5697.
- Martin-Laurent, F., Piutti, S., Hallet, S., Wagschal, I., Philippot, L., Catroux, G. and Soulas, G. (2003) Monitoring of atrazine treatment on soil bacterial, fungal and atrazine-degrading communities by quantitative competitive PCR. *Pest Manag Sci* 59, 259–268.
- Mulbry, W.W., Zhu, H., Tour, S.M. and Topp, E. (2002) The triazine hydrolase gene *trzN* from *Nocardioides* sp. strain C190: cloning and construction of gene-specific primers. *FEMS Microbiol Lett* 206, 75–79.
- Newcombe, D.A. and Crowley, D.E. (1999) Bioremediation of atrazine-contaminated soil by repeated applications of atrazine-degrading bacteria. *Appl Microbiol Biotechnol* 51, 877–882.
- Pesaro, M., Nicollier, G., Zeyer, J. and Widmer, F. (2004) Impact of soil drying-rewetting stress on microbial communities and activities and on degradation of two crop protection products. *Appl Environ Microbiol* 70, 2577–2587.
- Piutti, S., Semon, E., Landry, D., Hartmann, A., Dousset, S., Lichtfouse, E., Topp, E., Soulas, G. *et al.* (2003) Isolation and characterization of *Nocardioides* sp. SP12, an atrazine-degrading bacterial strain possessing the gene *trzN* from

- bulk- and maize rhizosphere soil. *FEMS Microbiol Lett* 221, 111–117.
- Ralebitso, T.K., Senior, E. and van Verseveld, H.W. (2002) Microbial aspects of atrazine degradation in natural environments. *Biodegradation* 13, 11–19.
- Rees, G.N., Baldwin, D.S., Watson, G.O., Perryman, S. and Nielsen, D.L. (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Antonie Van Leeuwenhoek* 86, 339–347.
- Rhine, E.D., Fuhrmann, J.J. and Radosevich, M. (2003) Microbial community responses to atrazine exposure and nutrient availability: linking degradation capacity to community structure. *Microb Ecol* 46, 145–160.
- Rousseaux, S., Soulas, G. and Hartman, A. (2001) Isolation and characterization of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiol Ecol* 36, 211–222.
- Rousseaux, S., Soulas, G. and Hartmann, A. (2002) Plasmid localization of atrazine-degrading genes in newly described *Chelatobacter* and *Arthrobacter* strains. *FEMS Microbiol Ecol* 41, 69–85.
- Runes, H.B., Jenkins, J.J., Moore, J.A., Bottomley, P.J. and Wilson, B.D. (2003) Treatment of atrazine in nursery irrigation runoff by a constructed wetland. *Water Res* 37, 539–550.
- Sajjaphan, K., Shapir, N., Wackett, L.P., Palmer, M., Blackmon, B., Tomkins, J. and Sadowsky, M.J. (2004) *Arthrobacter aurescens* TC1 atrazine catabolism genes *trzN*, *atzB*, and *atzC* are linked on a 160-kilobase region and are functional in *Escherichia coli*. *Appl Environ Microbiol* 70, 4402–4407.
- Sánchez, M., Garbí, C., Martínez-Alvarez, R., Ortiz, L.T., Allende, J.L. and Martín, M. (2005) *Klebsiella planticola* strain DSZ mineralizes simazine: physiological adaptations involved in the process. *Appl Microbiol Biotechnol* 66, 589–596.
- Santiago-Mora, R., Martin-Laurent, F., de Prado, R. and Franco, A.R. (2005) Degradation of simazine by microorganisms isolated from soils of Spanish olive fields. *Pest Manag Sci* 61, 917–921.
- Seffernick, J.L. and Wackett, L.P. (2001) Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. *Biochemistry* 40, 12747–12753.
- Seffernick, J.L., de Souza, M.L., Sadowsky, M.J. and Wackett, L.P. (2001) Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different. *J Bacteriol* 183, 2405–2410.
- Shao, Z.Q., Seffenis, W., Mulbry, W. and Behki, R.M. (1995) Cloning and expression of the s-triazine hydrolase gene (*trzA*) from *Rhodococcus corallinus* and development of *Rhodococcus* recombinant strains capable of dealkylating and dechlorinating the herbicide atrazine. *J Bacteriol* 177, 5748–5755.
- Shapir, N., Goux, S., Mandelbaum, R.T. and Pussemier, I. (2000) The potential of soil microorganisms to mineralize atrazine as predicted by MCH-PCR followed by nested PCR. *Can J Microbiol* 46, 425–432.
- Silva, E., Fialho, A.M., Sa-Correia, I., Burns, R.G. and Shaw, L.J. (2004) Combined bioaugmentation and biostimulation to cleanup soil contaminated with high concentrations of atrazine. *Environ Sci Technol* 38, 632–637.
- Singh, P., Suri, C.R. and Cameotra, S.S. (2004) Isolation of a member of *Acinetobacter* species involved in atrazine degradation. *Biochem Biophys Res Commun* 317, 697–702.
- de Souza, M.L., Sadowsky, M.J. and Wackett, L.P. (1996) Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. *J Bacteriol* 178, 4894–4900.
- de Souza, M.L., Seffernick, J., Martinez, B., Sadowsky, M.J. and Wackett, L.P. (1998a) The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J Bacteriol* 180, 1951–1954.
- de Souza, M.L., Wackett, L.P. and Sadowsky, M.J. (1998b). The *atzABC* genes encoding atrazine catabolism are located on a self-transmissible plasmid in *Pseudomonas* sp. strain ADP. *Appl Environ Microbiol* 64, 2323–2326.
- Strandberg, M.T. and Scott-Fordsmand, J.J. (2002) Field effects of simazine at lower trophic levels – a review. *Sci Total Environ* 296, 117–137.
- Stroetmann, I., Kampfer, P. and Dott, W. (1994) The efficiency of sterilization methods for different soils. *Zentralbl Hyg Umweltmed* 195, 111–120.
- Strong, L.C., McTavish, H., Sadowsky, M.J. and Wackett, L.P. (2000) Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. *Environ Microbiol* 2, 91–98.
- Struthers, J.K., Jayachandran, K. and Moorman, T.B. (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *Appl Environ Microbiol* 64, 3368–3375.
- Top, E.M., Holben, W.E. and Forney, L.J. (1995) Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. *Appl Environ Microbiol* 61, 1691–1698.
- Topp, E., Zhu, H., Nour, S.M., Houot, S., Lewis, M. and Cuppels, D. (2000) Characterization of an atrazine-degrading *Pseudaminobacter* sp. isolated from Canadian and French agricultural soils. *Appl Environ Microbiol* 66, 2773–2782.
- Tuxen, N., de Liphay, J.R., Albrechtsen, H.J., Aamand, J. and Bjerg, P.L. (2002) Effect of exposure history on microbial herbicide degradation in an aerobic aquifer affected by a point source. *Environ Sci Technol* 36, 2205–2212.
- Wackett, L.P., Sadowsky, M.J., Martinez, B. and Shapir, N. (2002) Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. *Appl Microbiol Biotechnol* 58, 39–45.

- Watanabe, K., Futamata, H. and Harayama, S. (2002) Understanding the diversity in catabolic potential of microorganisms for the development of bioremediation strategies. *Antonie Van Leeuwenhoek* 81, 655–663.
- Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K. and De Ley, J. (1992) Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *Int J Syst Bacteriol* 42, 107–119.
- Yanze-Kontchou, C. and Gschwind, N. (1994) Mineralization of the herbicide atrazine as a carbon source by a *Pseudomonas* strain. *Appl Environ Microbiol* 60, 4297–4302.
- Zhao, S., Arthur, L.E. and Coats, J.R. (2003) Influence of microbial inoculation (*Pseudomonas* sp. strain ADP), the enzyme atrazine chlorohydrolase, and vegetation on the degradation of atrazine and metolachlor in soil. *J Agric Food Chem* 51, 3043–3048.