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STUDY OF MICROBIAL COMMUNITIES IN SIMAZINE TREATED AGRICULTURAL SOILS AND THEIR BIOTECHNOLOGICAL POTENTIAL

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STUDY OF MICROBIAL COMMUNITIES IN SIMAZINE TREATED AGRICULTURAL SOILS AND THEIR BIOTECHNOLOGICAL POTENTIAL

Esta tesis es presentada bajo la supervisión del Director de Tesis, Dr. MICHAEL SEEGER PFEIFFER, del departamento de Química de la Universidad Técnica Federico Santa María para su aprobación por la comisión.

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Mamá, te dedico mi tesis
por ser el orgullo de mi vida
y el motor en mi diario desafío
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STUDY OF MICROBIAL COMMUNITIES IN SIMAZINE TREATED AGRICULTURAL SOILS AND THEIR BIOTECHNOLOGICAL POTENTIAL

Abstract

s-Triazine herbicides are used in agriculture and forestry in diverse regions of the world. The pollution of the environment with *s*-triazines is of increasing concern due to their toxicity and wide distribution. Simazine has been one of the most used herbicides in Chile. The simazine application in agricultural soils can potentially lead to contamination of water bodies. Although, natural dissipation of *s*-triazines in soils by physicochemical processes has been described, the main mechanism for their removal is the microbial degradation. The *atzA, atzB, atzC, atzD, atzE* and *atzF* genes encode the enzymes for the mineralization of *s*-triazines in diverse bacteria. A variety of culture-independent molecular methods, such as denaturing gradient gel electrophoresis (DGGE) have been used for monitoring the spatial and temporal composition of soil microbial communities. However, the effect of herbicides on soil microbial communities as well as their potential role in the degradation of herbicides still remains to be elucidated. The aims of this study were to characterize and to identify *s*-triazine-degrading bacteria, to detect *s*-triazine catabolic genes in those bacteria, to study the short-term effects of simazine on the *Bacteria, Fungi* and *Archaea* in agricultural soils and to analyse the influence of simazine on the nitrification process and on the ammonia-oxidizing bacteria (AOB) and the ammonia-oxidizing archaea (AOA). Diverse strains isolated from agricultural soils of central Chile and able to degrade simazine were characterized. Selected bacteria were characterized by microbiological and biochemical test and by sequencing 16S rRNA genes as well as housekeeping genes. Bacteria were identified as species of *Pseudomonas*, *Stenotrophomonas* and *Arthrobacter*. Multi locus sequence analyses (MLSA) were used in this study for further bacterial characterization, especially with *Pseudomonas* and *Stenotrohpomonas* strains. The strain MHP41 was the the most efficient *s*-triazine degrading bacterium of the isolates. This strain was able to grow in minimal medium using simazine as the sole nitrogen source. Resting cells of strain MHP41 degraded more than 80% of simazine within 60 min. The catabolic *atzA, atzB, atzC, atzD, atzE* and *atzF* genes

were detected in strain MHP41 and *Stenotrophomas spp.* strains P13, P33, P43 and C53. Strain MHP41 was initially allocated in the genus *Pseudomonas*, by phenotypic (Biolog) and 16S rRNA gene sequence analyses. Physiological and biochemical characterizations of strain MHP41 indicated that it is closely related with *P. multiresinovorans* and *P. nitroreducens*. The analyses of the data obtained with the technique MLSA, by 16S rRNA, *gyr*B and *rpoB* housekeeping genes, as well as 16S-23S rRNA inter-genic spacer (IGS-1) region sequencing, further identified strain MHP41 as *Pseudomonas nitroreducens*. Strain MHP41 and the other strains characterized in the thesis could be potential biocatalysts for bioremediation processes to remove *s*-triazines from polluted environments. The short-term effects of simazine on soil microbial community composition was evaluated by DGGE of PCR-amplified 16S rRNA and 18S rRNA gene fragments. After simazine application in soils, bacterial groups closely related to *Pseudomonas* and *Acinetobacter* genera were stimulated, independently to the previous history of simazine application in the soils. Although, fungal communities were only slightly affected by simazine application, this herbicide stimulated fungi clones closely related to *Paecilomyces* and *Penicillium* genera in soils with history of simazine application. Simazine promoted changes on the composition of archaeal communities in both soils. Main changes were related to members of Crenarchaeota. This study indicated that bacterial and archaeal communities are mainly affected by simazine application in agricultural soils. Finally, the effect of simazine on nitrification process in soil was also studied. DGGE patterns showed that AOB in the agricultural soil was affected by the simazine incubation. However, simazine did not affect AOA. These results indicated that simazine induced changes in AOB rather than AOA in an agricultural soil. In conclusions, in this study, the efficient *s*-triazine-degrading bacterium *Pseudomonas nitroreducens* strain MHP41 was characterized and identified. Other Gram-negative strains belonging to *Arthrobacter* and *Stenotrophomonas* genera were also characterized and identified. In this thesis, culture-dependent and culture-independent methods were used for the analysis of *s*-triazine degrading strains and to evaluate the effect of the herbicide simazine on microbial communities in agricultural soils.

ESTUDIO DE COMUNIDADES MICROBIANAS EN SUELOS AGRÍCOLAS TRATADOS CON SIMAZINA Y SU POTENCIAL BIOTECNOLÓGICO

Resumen

Los herbicidas de la familia de las *s*-triazinas son empleados en la agricultura y la silvicultura en diversas regiones del mundo. La contaminación del medio ambiente con *s*-triazinas ha generado una creciente preocupación, debido a su toxicidad y amplia distribución. La simazina es uno de los herbicidas más empleados en Chile. Su aplicación en los suelos agrícolas puede conducir a la contaminación de los cuerpos de agua. Si bien, la disipación natural de *s*-triazinas en los suelos por los procesos físico-químicos se ha descrito, el principal mecanismo para su eliminación es la degradación microbiológica. Los genes *atzA, atzB, atzC, atzD atzE* y *atzF* codifican las enzimas para la mineralización de *s*-triazinas en diversas bacterias. Una variedad de métodos moleculares cultivo-independientes, como la electroforesis en gel con gradiente de desnaturalización (DGGE) se utilizan para monitorear la composición espacial y temporal de las comunidades microbianas del suelo. Sin embargo, el efecto de los herbicidas sobre las comunidades microbianas del suelo, así como su papel potencial en la degradación de los herbicidas aún queda por dilucidar. Los objetivos de este estudio fueron caracterizar e identificar las bacterias degradadoras de *s*-triazina, la determinación de los genes catabólicos de *s*-triazinas de estas bacterias, estudiar los efectos a corto plazo de simazina sobre *Bacteria, Archaea* y *Fungi* en los suelos agrícolas y analizar la influencia de la simazina sobre los procesos de nitrificación y en las comunidades bacterias amonio-oxidasas (AOB) y las arqueas amonio-oxidasas (AOA). Diversas cepas aisladas desde los suelos agrícolas de Chile central y capaces de degradar simazina fueron caracterizadas. Las bacterias seleccionadas fueron caracterizadas mediante pruebas microbiológicas y bioquímicas y por secuenciación del gen 16S rRNA y por otros genes de manutención celular. Las bacterias fueron identificadas como *Pseudomonas, Stenotrophomonas* y *Arthrobacter*. El análisis de las secuencias multilocus (MLSA) fue empleada en este estudio para la caracterización bacteriana en *Pseudomonas* y *Stenotrohpomonas*. La cepa MHP41 degradó más del 80% de la simazina en 60 min de exposición. Los genes catabólicos *atzA, atzB, atzC, atzD, atzE* y *atzF* fueron detectados en la cepa MHP41 y en *Stenotrophomas* spp. cepas P13, P33, P43 y

C53. La caracterización fisiológica y bioquímica de la cepa MHP41 indicó una cercana relación con *P. multiresinovorans* y *P. nitroreducens*. El análisis de los datos obtenidos con la técnica MLSA, mediante la secuenciación de los genes 16S rRNA, *gyrB* y *rpoB*, así como la región intergénica 16S-23S rRNA (IGS-1), identificaron a la cepa MHP41 como *Pseudomonas nitroreducens*. La cepa MHP41 así como las otras cepas caracterizadas en esta tesis podrían ser potenciales biocatalizadores para los procesos de biorremediación tendientes a la remoción de los herbicidas *s*-triazinas desde los ambientes contaminados. Para evaluar los efectos a corto plazo de la simazina sobre la composición de la comunidad microbiana en los suelos agrícolas, se realizó DGGE de los genes del ARN ribosomal 16S y ARN ribosomal 18S. La incubación con simazina en los suelos estimuló grupos bacterianos relacionados con los géneros *Pseudomonas* y *Acinetobacter*, independiente del historial de aplicación simazina en los suelos. Aunque, las comunidades de hongos fueron sólo ligeramente afectados por la aplicación simazina, este herbicida estimuló hongos estrechamente relacionados con los géneros *Paecilomyces* y *Penicillium* en los suelos con historial de aplicación de la simazina. La simazina promovió cambios en la composición de las comunidades de las arqueas en ambos suelos. Los principales cambios ocurrieron en la clase Crenarchaeota. Este estudio indicó que las comunidades de bacterias y arqueas son los más afectados por la aplicación de la simazina en los suelos agrícolas. Finalmente, se estudió el efecto de simazina sobre los procesos de nitrificación. Los patrones de DGGE mostraron que la incubación con simazina afectó las AOB en los suelos agrícolas. Sin embargo, simazina no afectó las AOA. Estos resultados indicaron que simazina promovió cambios en las AOB pero no en las AOA en el suelo agrícola. En este estudio, se caracterizó e identificó la cepa *Pseudomonas nitroreducens* MHP41, una bacteria degradadora de *s*-triazina eficiente. Asimismo, se caracterizaron e identificaron otras cepas Gram-negativas pertenecientes a los géneros *Arthrobacter* y *Stenotrophomonas*. En la presente tesis, se emplearon métodos de cultivo dependiente y métodos de cultivo independiente para el análisis de cepas degradadoras de *s*-triazinas para evaluar el efecto del herbicida simazina sobre las comunidades microbianas en suelos agrícolas.

List of abbreviations

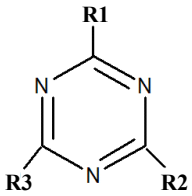
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AM	atrazine medium
ARDRA	amplified ribosomal DNA restriction analysis
BSA	bovine serum albumin
CCUG	Culture Collection, University of Göteborg
CFU	colony forming unit
CTAB	hexadecyl trimethyl-ammonium bromide
DGGE	denaturing gradient gel electrohporesis
LD	letal dosis
DNA	desoxyribonucleic acid
EDTA	ethylendiamine tetra-acetic acid
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
FISH	fluorescent *in situ* hybridization
gyrB	gyrase β-subunit
HPLC	high-performance liquid chromatography
IGS	inter-genic spacer
LB	luria bertani
MLSA	multi-locus sequence analyses
MPN	most-probable-number
NCBI	National Center for Biotechnology Information
OD_{600}	optical densityof 600 nm
PCR	polymerase chain reaction
RISA	ribosomal intergenic spacer analysis
*rpo*B	RNA polymerase β-subunit
rpoD	RNA polymerase sigma factor
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAG	Servicio Agrícola y Ganadero
SSCP	single strand conformation polymorphism
TBE	tris borate EDTA
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
TSA	tryptic soy agar
TS	tryptic soy
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
WHC	water-holding capacity

Chapter 1

General Introduction

Introduction

s-Triazine herbicides are used for the control of broad leaf and grassy weeds in many regions of the word (Wackett *et al.*, 2002). *s*-Triazines are xenobiotic compounds that are released in large amounts in the environment. *s*-Triazines are strong inhibitors of photosynthesis in annual grasses and broadleaf weeds, interrupting the electron transport chain in photosystem II (Gunasekara *et al.*, 2007). These compounds inhibit the growth of many weeds. Atrazine and simazine are the most important *s*-triazine herbicides**.** Atrazine has been used for over 40 years and is one of the most used herbicide in United States (EPA, 2004). Simazine was first introduced in USA in 1956 and has been widely used for preemergence control (Gunasekara *et al.*, 2007). Other *s*-triazine herbicides include propazine, terbutylazine, deethylatrazine and cyanazine (Fig. 1).



Substituent			Chemical formula	Common name
R1	**R2**	**R3**		
Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	2-Chloro-4,6-bis(ethylamino)-*s*-triazine	Simazine
OH	$NHCH_2CH_3$	$NHCH_2CH_3$	2-Hydroxy-4,6-bis(ethylamino)-*s*-triazine	Hidroxysimazine
OH	NH_2	$NHCH_2CH_3$	2-Hydroxy-4-amino-6- ethylamino-*s*-triazine	Deetilhidroxisimazina
Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	2-Chloro-4-ethylamino-6-isopropylamino-*s*-triazine	Atrazine
OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	2-Hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine	Hidroxyatrazine
Cl	NH_2	$NHCH(CH_3)_2$	2-Chloro,4-amino-6-isopropylamino-*s*-triazine	Deethylatrazine
OH	NH_2	$NHCH(CH_3)_2$	2-Hydroxy-4-amino-6-isopropylamino-*s*-triazine	Deethylhydroxyatrazine
Cl	$NHCH_2CH_3$	NH_2	2-Chloro-4-ethylamino-6-amino-*s*-triazine	Deisopropylatrazine
Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	2-Chloro-4,6-bis(isopropylamino)-*s*-triazine	Propazine
Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$	2-Chloro-4-ethylamino-6-terbutylamino-*s*-triazine	Terbutylazine
OH	OH	OH	2,4,6-Trihydroxy-*s*-triazine	Cianuric acid
Cl	$NHCH_2CH_3$	$NHC(CH_3)_2CN$	2-[(4-Chloro-6-ethylamino-*s*-triazine-2-yl)amino]-2-methyl propionitrile	Cyanazine

Figure. 1. Chemical structures of *s*-triazines Members of *s*-triazine herbicides differ by their R_1, R_2 and R_3 groups of the *s*-triazine ring.

In Chile an important increase of pesticides consumption has been observed in the last years. The herbicide consumption has increased in Chile, from 4000 ton in 2004 to 8000 ton in 2006 (SAG, 2004; 2006). Simazine and atrazine are the most applied herbicides, mainly for grass control in agriculture and forestry, with a consumption around 219 tons of simazine in 2004 (SAG, 2006).

_s_-Triazine persistence.

Several factors determine the persistence of herbicides in soil. Chemical soil properties such as soil texture, organic matter content as well as the pH influence herbicide persistence. Chemical properties of the herbicide such as solubility, vapor pressure, and the molecule susceptibility to chemical or microbial degradation determine the environmental stability of the compound (Kogan, 1993; Gunasekara *et al.*, 2007). Climatic variables, mainly moisture and temperature, are also relevant factors (García-Valcárcel and Tadeo, 1999; Tappe *et al.,* 2002). At high temperatures (>70ºC) atrazine hydrolyses to hydroxyatrazine (Tappe *et al*., 2002). Finally, microbial abundance and metabolic activities of soil microorganisms play a central role on herbicide fate in soil (Aislabie and Lloydjones, 1995; Prosser *et al*., 2007; Seeger *et al*., 2010).

The retention of *s*-triazines herbicides by soil constituents has been described. Soil organic matter and clay minerals are effective sorbents for *s*-triazines (García-Valcárcel and Tadeo, 1999; Flores *et al.*, 2009). Therefore, physicochemical characteristics of the herbicide, the active surface of the minerals and the organic content of soils as well as the amount of herbicide applied are important parameters for understanding the dynamics of *s*-triazine herbicides in the environment. To protect ground- and surface waters from pesticide contamination, and to ameliorate their impact, broad knowledge is required concerning their sorption-desorption processes and biodegradation in the environment. García-Valcárcel and Tadeo (1999) evaluated the influence of soil moisture on sorption of simazine in soil. Soils incubated, during 44 days, at 25 °C with moisture contents ranging from 4% to 18%, showed sorption values (K_f) for simazine ranging from 0.5 to 1.2. Herbicide degradation rates increased with soil moisture content, and drying−rewetting of soil yielded degradation rates slower than that obtained at 10% soil moisture content.

_s_-Triazine pollution and toxicity.

s-Triazine herbicides can cause serious problems in the ecosystems and human health (Foth, 1999). *s*-Triazines can cause severe damages to organisms during the exposure through food, water and air (Newcombe *et al.*, 2002). The *s*-triazines atrazine, simazine and cyanazine were classified by EPA as a "possible human carcinogen" of class C (Tappe *et al.*, 2002). Atrazine induces mammary cancer in female rats (Birnbaum and Fenton, 2003). The toxic concentration of atrazine LD_{50} is 3.0 g/kg in rats and 1.75 g/kg in mice (Yanze-Kontchou and Gschwind, 1999). The possible role of *s*-triazines in the promotion of human breast cancer has been reported (Kettles *et al.*, 1997). Chromosomal damage has been observed in human lymphocytes exposed to atrazine (Roloff *et al.*, 1992). *s*-Triazines are potent endocrine disruptors and provoke reproductive disorders in mammalians (Fan *et al.*, 2007).

Atrazine at concentrations over 0.1 µg/l induce hermaphroditism in American Leopard frogs and at concentration higher than 1.0 µg/l demasculinized the larynges of exposed males (Hayes *et al.*, 2002; Hayes *et al.*, 2003).

The maximum acceptable levels of *s*-triazine herbicides for drinking water are 0.1 µg/L in Europe and 3.0 µg/l in the USA (Rousseaux *et al.*, 2001). Atrazine has been detected in surface water resources, which receive drainage from intensively farmed agricultural production areas. These areas contain higher levels of pesticides, particularly after pesticide applications (Radosevich *et al.*, 1995). In Germany, atrazine application has been forbidden since 1991. However, high concentrations of atrazine and desethylatrazine were still found in groundwater in 2002. (Tappe *et al.*, 2002). Atrazine has been detected in rain water (mainly at concentrations below 1.0 µg/l) and close to treated areas (Tappe *et al*., 2002).

The detection of atrazine and other pesticides in lakes and watershed in central-south Chile causes increasing concern (Cooman *et al.*, 2005). Atrazine has been detected in groundwater (Garrido *et al.*, 1998; Papiernik and Spalding, 1998; Spalding *et al.*, 2003) at levels which frequently exceed the maximum acceptable levels of *s*-triazine herbicides for drinking water by the U.S. Environmental Protection Agency (EPA). Therefore, atrazine represents a direct risk for human health via potable water consumption (Aislabie and

Lloydjones, 1995; Boundy-Mills *et al.*, 1997). To minimize the damages produced by these herbicides, processes for the removal of *s*-triazines from the polluted sites have been studied.

Bacterial degradation of *s*-triazines

s-Triazines can be degraded by physicochemical processes, such as oxidation, photolysis and hydrolysis (Gunasekara *et al.*, 2007; Flores *et al.*, 2009). However, microbial degradation is the main mechanism for the removal of these compounds from the environment (Aislabie and Lloydjones, 1995; Seeger *et al*., 2010). Diverse bacteria (Cook and Hütter, 1881; Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995; Struthers *et al.*, 1998; Rousseaux *et al.*, 2001; Piutti *et al.*, 2003; Iwasaki *et al.*, 2007; Hernández *et al.*, 2008), fungi (Singh *et al.*, 2004; Singh *et al.*, 2008) and mixed communities of microorganisms (Newcombe and Crowley, 1999; Topp, 2001) capable of degrade atrazine have been isolated.

The *s*-triazine pathways of bacteria have been studied (Fig. 2). The upper pathway of simazine degradation involves three enzymes codified by *atzA*, *atzB* and *atzC* genes. The first enzyme of the catabolic pathway is atrazine chlorohydrolase, which catalyzes simazine dechlorination to produce hidroxysimazine. This enzyme is codified by the *atzA* gene. The enzymes hydroxyatrazine ethylaminohydrolase and N-isopropylammelide isopropylaminohydrolase convert sequentially hidroxysimazine to N-ethylammelide and to cyanuric acid (Boundy-Mills *et al.*, 1997; De Souza *et al.*, 1998; Sadowsky *et al.*, 1998). These hydrolases are codified by the *atzB* and *atzC* genes. AtzA, AtzB and AtzC enzymes are members of the amidohydrolase protein superfamily (Janssen *et al.*, 2005). Amidohydrolases are present mainly in microorganisms, but also in plants and animals, playing an important role in the mineralization of nitrogen containing compounds and supply of nitrogen for the plants (Strong *et al.*, 2002; Wackett *et al.*, 2002). Other members of amidohydrolase superfamily are triazine deaminase, melamine deaminase, cytosine deaminase and phosphotriesterase (Janssen *et al.*, 2005). The enzyme melamine deaminase (TriA) from *Pseudomonas* sp. strain NRLLB-12227 is 98% identical to atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. ADP, suggesting a recent evolutionary divergence (Seffernick *et al.*, 2001). The lower pathway of simazine degradation includes the enzymes cyanuric acid amidohydrolase, biuret hydrolase and allophanate hydrolase,

which are codified by the *atzD, atzE* and *atzF* genes, respectively. These enzymes transform the compound cyanuric acid through biuret and alophanate into carbon dioxide and ammonium (Strong *et al.*, 2002; Cheng *et al.*, 2005; Shapir *et al.*, 2005; Kolic *et al.*, 2007).

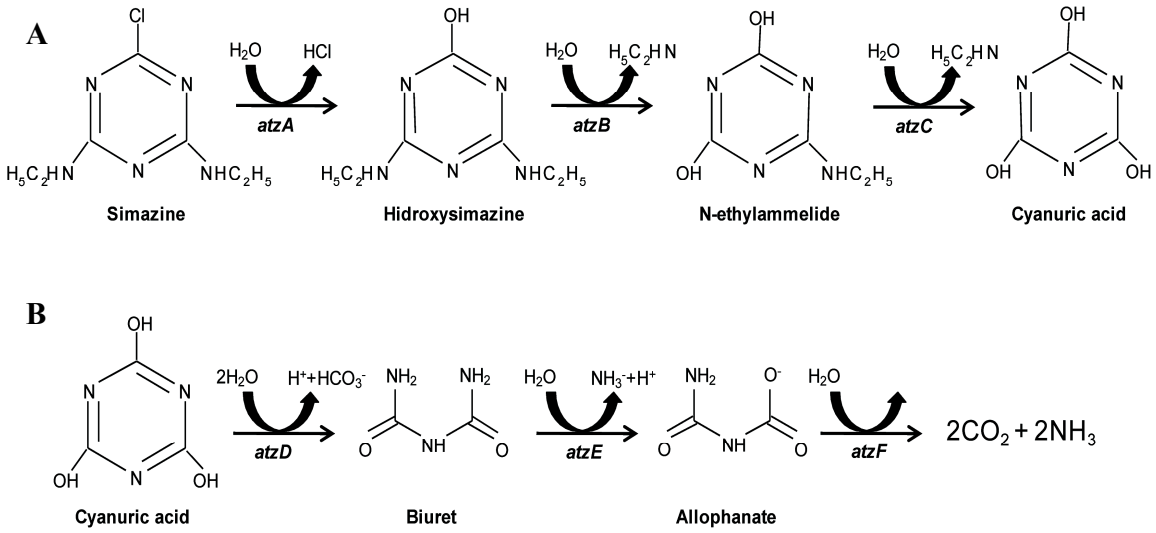


Figure 2. The simazine catabolic pathway. A, Upper catabolic pathway. B, Lower catabolic pathway.

Only few bacterial strains posses the six *atz* genes. In *Pseudomonas* sp. ADP all six *atz* genes are localized on the 100 kb plasmid pADP-1 (Martínez *et al.*, 2001). Strain CDB21 and *Agrobacterium* sp. NEA-D posses all the *atz* genes for the simazine degradation (Devers *et al*., 2007a; Iwasaki *et al.*, 2007). Most of these bacteria are capable to degrade one or more related *s*-triazines. These *atz* genes are highly conserved (>97% similarity) in a broad range of bacteria suggesting their recent dispersion within the soil microflora (De Souza *et al.*, 1998; Rousseaux *et al.*, 2001; Devers *et al.*, 2007b). However, only relatively few strains can completely mineralize these herbicides. A relationship between the presence of all *atz* genes and the mineralization capabilities of bacterial strains

has been described (Rousseaux *et al.*, 2001; Santiago-Mora *et al.*, 2005; Devers *et al.*, 2007a).

Recently, novel genes involved in *s*-triazine degradation have been reported and designed as *trz* genes. An alternative atrazine-degrading pathway in Gram-positive was first studied in *Nocardioides* sp. C190 based in the discovery of a novel hydrolase, TrzN. This enzyme is characterized by a broader substrate specificity than that of the AtzA from *Pseudomonas* sp. ADP (Kolic *et al.*, 2007). The *trzN* gene was also detected in diverse *s*-triazine-degrading strains (Devers *et al.*, 2007a). The *trzD* gene, which encodes the enzyme responsible for the *s*-triazine ring cleavage of cyanuric acid, was also found in *Pseudomonas* sp. NRRLB-122227 (Kolic *et al.*, 2007), and in other *s*-triazine-degrading strains (Rousseaux *et al.*, 2001; Fruchey *et al.*, 2003; Devers *et al.*, 2007a). *Nocardioides* sp. strain SP12 has a novel atrazine upper catabolic pathway, which enzymes are encoded by *trzN, atzB* and *atzC* genes (Piutti *et al.*, 2003; Shapir *et al.*, 2007). This pathway is also present in the atrazine-degrading strains *Arthrobacter cristallopoietes* Cit2, *Sinorhizobium* sp. strain NEA-B and *Polaromonas* sp. strain NEA-C (Devers *et al.*, 2007a).

The *atz* and *trz* genes were often located on plasmids and associated to transposons, suggesting that horizontal gene transfer is important in their dispersion. As diverse *s*-triazine degrading microbes have several catabolic *atz* and *trz* combinations, these genes seem to be the result of a recent evolution (Seeger *et al.*, 2010).

Microbial communities in *s*-triazine treated soils

The study of microbial communities in an herbicide-contaminated soil is crucial to understand the herbicide biodegradation and to design bioremediation processes. The integration of culture-dependent and culture-independent methods enables monitoring the microbial communities inhabiting the soil ecosystem as well as the microorganisms responsible for *s*-triazine catabolism (Seeger *et al.*, 2010).

Microorganisms play key functions in geochemical cycles in soil. For the fate of herbicides in soil the microbial communities are crucial. Microorganisms are involved in herbicide degradation and are the catalysts for bioremediation processes. The cultivation of soil microorganisms has been very useful to study the microbial metabolism, genetics and

physiology. The development of culture-independent methods enables characterizing in the structure and function of microbial communities in soil. The molecular biology tools allow circumventing the requirement for cultivation in the characterization of the microbial diversity in an environment. The structure of microbial communities could be studied by the analysis of specific gene sequences. The 16S ribosomal RNA gene sequence analysis is generally used for studying the diversity of microbial communities in the environment. The 16S rRNA gene sequence analysis of the microbial community by molecular approaches such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998; Muyzer, 1999), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997) or clone libraries (Liesack and Stackebrandt, 1992) indicate the biodiversity and the structure of microbial communities in the environment. In addition, fluorescent *in situ* hybridization (FISH) (Morgante *et al.*, 2010) uses fluorochrome-labeled oligonucleotides to identify *in situ* specific active microbial communities, obtaining direct measures of their relative abundance. These molecular methods have been used for the analyses of the microbial communities of pesticide-treated soils and the dynamics of microbial communities during bioremediation (Paul *et al.*, 2006; Morgante *et al.*, 2010). Culture-independent methods methodologies have revealed that the majority of soil microorganisms have not been cultured in the laboratory (Saito *et al*., 2007; Torsvik *et al.*, 1990). The knowledge of the structure and function of microbial communities of *s*-triazine contaminated soils by culture-independent methods helps to understand the adaptation of the microorganisms to the pesticides and to identify the microbes involved in their degradation.

In atrazine-treated agricultural soils, long term application of organic amendment changes the microbial community (Martin-Laurent *et al.*, 2004). The addition of atrazine and inorganic nitrogen source increases atrazine-degrading microorganism (Rhine *et al.*, 2003). More recently, it has been reported that bioaugmentation with strain MHP41 changes microbial communities in simazine adapted and non-adapted agricultural soils (Morgante *et al.*, 2010). FISH analysis reported by Morgante *et al.* (2010) revealed that bioaugmentation increased the relative abundances of few bacterial groups in soils. The bacterial community response could be a consequence of the enhanced nitrogen

bioavailability after simazine bioremediation (Cook and Hütter, 1881; Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995; Rhine *et al.*, 2003).

Effects of *s*-triazine on nitrification processes

Nitrification involves the biological oxidation of ammonia to nitrate and is of fundamental importance in the global nitrogen cycle (Fig. 3). The nitrification potential is strongly affected by physico-chemical parameters such as soil pH (Nicol *et al.*, 2008), temperature (Avrahami *et al.*, 2003; Tourna *et al.*, 2008) and availability of CO_2 (Azam *et al.*, 2004).

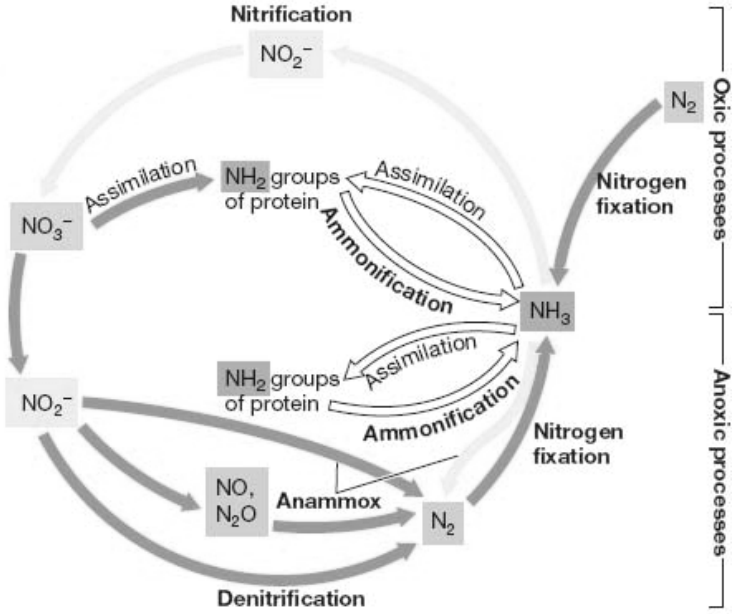


Figure 3. Redox cycle for nitrogen. Oxidation reactions are shown by light grey arrows, reductions reactions are shown by dark grey arrows. Reactions without redox change are in white arrows (modified from Madigan *et al*., 2009).

Recently, it has been reported that the *amoA* gene for the ammonia monooxygenase. gene occurs in both *Bacteria* and in *Archaea* (Avrahami *et al*., 2003; Jia and Conrad, 2009; Leininger *et al.*, 2006; Nicol *et al.*, 2008; Shen *et al.*, 2008; Treusch *et al*., 2005). Ammonia oxidizing bacteria (AOB) play an important role in the nitrogen cycle (Jia and Conrad, 2009; Shen *et al*., 2008). AOB involved in the aerobic oxidation of ammonia to nitrite

belong to the genus *Nitrosomas*. The bacteria responsible for the conversion of nitrite to nitrate belong to the genus *Nitrobacter* (Sahrawat, 2008). Quantitative analysis of the *amoA* genes showed that *Archaea* predominate among the ammonia-oxidizing prokaryotes in seawater (Karner *et al.*, 2001) and soil samples (Schleper *et al.*, 2005; Treusch *et al.*, 2005; Leininger *et al.*, 2006; Shen *et al.*, 2008).

Archaea play a significant role in ammonia oxidation and had a significant impact on the nitrification processes (Francis, 2007). Prosser and Nicol (2008) have reported that ammonia oxidizing archaea (AOA) are generally more abundant than the AOB, suggesting that *Archaea* may play an important role in nitrification in many environments. The archaeon *Nitrosopumilus maritimus*, recently isolated by Könneke *et al.* (2005) grows using ammonia as the sole energy source and converts ammonia to nitrite with concomitant increase in cell number. The potential for crenarchaeal ammonia oxidation has also been confirmed by enrichment and isolation of ammonia-oxidizing archaea from hot springs (Hatzenpichler *et al*., 2008; de la Torre *et al*., 2008). However, the precise nature of the AOA has not been elucidated and to date there are no isolates from soil. Fingerprints of AOB and AOA communities had been generally characterized by denaturing gradient gel electrophoresis (DGGE), which is an important tool for analyzing the sequence diversity of the complex natural microbial communities (Muyzer, 1999).

Only few studies have analyzed the effect of herbicides on the bacteria communities and on the nitrification process (Debona and Audus, 1970; Gaur and Misra, 1977; Li et al., 2008). Gaur and Misra (1977) have studied the effect of simazine, lindane and ceresan on the nitrification rates. Simazine (20 mg/kg) hampered the nitrification rate. However, after 35 days, nitrification was no further affected. Simazine at high concentration (200 mg/kg) inhibited nitrification during 35 days. Debona and Audus (1970) studied the effects of different herbicides on soil nitrification. The herbicide propanil caused serious and persistent decreases of nitrification in the field. Endothal stimulated ammonia oxidation and ioxynil and bromoxynil have a slight positive effect on the nitrification process. Recently, Li et al. (2008) have studied the impact of the widely used herbicide acetochlor in ammonia-oxidizing bacteria by DGGE., Acetochlor decreased the diversity of AOB after 60 days of incubation. The phylogenetic analysis revealed that strains closely related to members of the genus *Nitrosospira* were induced.

Several strong inhibitors of NH_3 oxidation in soil can be classified by their heterocyclic ring structures (McCarty, 1999). Compounds containing two or three non-adjacent ring containing N atoms, such as pyrimide, *s*-triazine, benzimidazole inhibits NH_3 oxidation in agricultural soils (McCarty and Bremner, 1989).

Hypothesis

The application of the herbicide simazine in agricultural soils promotes the presence of simazine-degrading bacteria and induces changes in the microbial community in soil and in the nitrification process.

General objective

To characterize *s*-triazine-degrading bacterial strains isolated from agricultural soils from central Chile and to evaluate the effect of the herbicide simazine on the microbial communities and the nitrification process in agricultural soils.

Specific objectives

1) To characterize and identify *s*-triazine-degrading bacterial strains isolated from agricultural soils from central Chile.

2) To detect *s*-triazine catabolic genes in bacterial strains isolated from agricultural soils from central Chile.

3) To study the effect of *s*-triazines application on the structure of microbial communities in an agricultural soils.

4) To study the effect of simazine application on the nitrification and on the structure of the ammonia-oxidizing bacteria and ammonia-oxidizing archaea in an agricultural soil.

Outline of the thesis

The research reported in the thesis was conducted in four research institutes: at the Laboratorio de Microbiología y Biotecnología Ambiental, Millennium Nucleus of Microbial Ecology and Environmental Microbiology and Biotechnology EMBA and Departamento de Química, Universidad Técnica Federico Santa María, Valparaíso, Chile and the Culture Collection University of Gothenburg (CCUG), Department of Clinical Bacteriology, Sahlgrenska University Hospital, Gothenburg, Sweden (Chapters 1-4), at the Center of Plant, Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile (Chapter 5) and at the Max-Planck-Institute for Terrestrial Microbiology, Marburg, Germany (Chapter 6).

The first two chapters of this study provide an overview of *s*-triazine-degrading bacteria and bioremediation processes (*Chapter 1: General introduction*; *Chapter 2: Modern approaches for the study of bioremediation of s-triazine herbicides in agricultural soils*).

The third and fourth chapters describe the research of the first two specific objectives of this thesis: Characterization and identification of *s*-triazine-degrading bacterial strains isolated from agricultural soils and detection of *s*-triazine catabolic genes in bacterial strains. Chapter three describes the physiological and biochemical characterizations and identification of *s*-triazine-degrading strains isolated, especially in the genus *Stenotrophomonas* (*Chapter 3, Part I: Novel s-triazine-degrading bacteria isolated from agricultural soils of central Chile for herbicide bioremediation*; *Chapter 3, Part II: Molecular characterization and phylogeny of novel herbicide-degrading Stenotrophomonas sp. strains isolated from Chilean agricultural soil*).

The fourth chapter is focussed in the physiological, biochemical and genetic characterization and detailed systematic classification of strain MHP41 by multi-locus sequence analyses (MLSA) *(Chapter 4, Part I: Isolation and characterization of a novel simazine-degrading bacterium from agricultural soil of central Chile, Pseudomonas sp. MHP41) (Chapter 4, Part II: Genetic and systematic characterization of an efficient simazine-degrading bacterium, Pseudomonas nitroreducens MHP41).*

The fifth chapter reports the study on the effect of *s*-triazines application on microbial communities in agricultural soils of central Chile. Microbial community compositions of the soils were analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and 18S rRNA genes *(Chapter 5: Short-term effects of the herbicide simazine on microbial communities in agricultural soils).*

The sixth chapter describes the effect of the herbicide simazine on the nitrification process and on the ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) *(Chapter 6: Effect of simazine application on nitrification processes and the microbial communities in agricultural soil)*.

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Chapter 2

Modern approaches for the study of *s*-triazine herbicide bioremediation in agricultural soils

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Abstract

The extensive use of *s*-triazine herbicides in diverse countries causes environmental and health concern. Simazine and atrazine are *s*-triazines widely used in agriculture and forestry. Although, natural dissipation of *s*-triazines in soils by physicochemical processes has been described, the main mechanism for their removal is biological degradation by microorganisms. Bioremediation is a successful strategy for the removal of *s*-triazines in soil. For bioaugmentation processes, *s*-triazine-degrading bacteria are required, which isolation from agricultural soils was described in this report. Studies of *s*-triazine adsorption and leaching in soil are useful to determine the bioavailability of these herbicides. The detection of *s*-triazine-degrading catabolic activity by most-probable-number (MPN) and the reduction of the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) were presented. The relative abundances of *s*-triazine catabolic genes in soil were analyzed by the MPN-PCR technique. Culture-independent molecular methods such as FISH, T-RFLP and clone libraries are useful to study the effects of herbicide application and bioaugmentation on soil microbial communities and their dynamics. These experimental methods allow the design of biotechnological strategies for the clean-up of *s*-triazine contaminated soils.

Resumen

El empleo masivo de herbicidas *s*-triazinas en diversos países ha causado preocupación ambiental y de salud. Simazina y atrazina son *s*-triazinas ampliamente utilizados en la agricultura y en predios forestales. La disipación natural de *s*-triazinas en suelos puede ocurrir por procesos físicoquímicos. Sin embargo, el principal mecanismo de remoción de estos herbicidas es la degradación mediada por microorganismos. La biorremediación es una estrategia eficiente para la remoción de *s*-triazinas del suelo. Para establecer procesos de bioaumentación, se requieren bacterias degradadoras de *s*-triazinas, cuyo aislamiento desde suelos agrícolas se describió en esta revisión. Estudios de adsorción y lixiviación de *s*-triazinas en suelos permiten determinar la biodisponibilidad de estos herbicidas. La actividad catabólica de microorganismos degradadores de simazina en suelo puede ser cuantificada por el método número más probable (NMP) y reducción del indicador de respiración cloruro de 2,3,5-trifenil-2H-tetrazolio (TTC). La abundancia

relativa de genes catabólicos de *s*-triazinas en suelo fue analizada mediante la técnica NMP-PCR. Técnicas moleculares cultivo independiente, tales como FISH, T-RFLP y librerías de clones, son útiles para estudiar los efectos de la aplicación de herbicidas y de la bioaumentación sobre la estructura de las comunidades microbianas del suelo y su dinámica. Los métodos experimentales descritos en esta revisión permiten el diseño de estrategias biotecnológicas eficientes para la recuperación de suelos contaminados con *s*-triazinas.

Introduction

s-Triazine herbicides, such as simazine and atrazine, have been used extensively for control of leaf and grassy weeds (Radosevich *et al.*, 1995). Due to the growing agricultural production in the world, large amounts of these herbicides have been applied. These agrochemicals, used primarily as pre- and post-emergent herbicides, interfere in the photosynthetic electron transport chain in susceptible plants by binding to the quinone-binding protein in photosystem II (Strong *et al.,* 2002). The mobility of *s*-triazine in soil has contributed to the contamination of surface- and groundwater in several countries (Mandelbaum *et al*., 1995; Radosevich *et al.*, 1995). Frequently, *s*-triazine herbicides have been detected exceeding the maximum pesticide acceptable levels in drinking water of Europe (0.1 $\mu g\ l^{-1}$) and USA (3.0 $\mu g\ l^{-1}$) (Mandelbaum *et al.,* 1995; Rousseaux *et al.*, 2001). In Chile, cultivation areas have been increased 70% between 1997 and 2007 (Instituto Nacional de Estadísticas, Chile, 2007. Censos Agropecuarios 1997 - 2007. www.ine.cl). Atrazine and simazine are the *s*-triazines herbicides most used in Chile, with an annual application of 181 and 169 tons, respectively (Servicio Agrícola Ganadero, 2004. Declaración de ventas de plaguicidas de uso agrícola. Ministerio de Agricultura. Santiago, Chile). Simazine is applied in vineyards, avocado, apple and maize plantations as well as in pine and eucalyptus plantations (Cooman *et al.,* 2005). Continuous application of *s*-triazines in Chilean soils is of increasing concern due to their potential contamination of surface- and groundwater. However, only few reports describe *s*-triazine contamination in Chile. In soils simazine may leach to 90 cm depth depending of weather conditions and soil properties (Alister *et al.,* 2005). Atrazine was detected in Chillán river at concentrations that are toxic for *Daphnia* spp. (Cooman *et al.,* 2005). *s*-Triazine maximum contaminant

levels for drinking water have not yet been established in Chile (Norma Chilena para Agua Potable, NCh 409/1. Of. 2005; Instituto Nacional de Normalización, Chile, www3.inn.cl).

Simazine degradation in soil occurs predominantly by biological processes (Gebendinger and Radosevich, 1998; Newcombe and Crowley, 1999). For the design of herbicide bioremediation processes in contaminated soils, the isolation of native bacteria and the characterization of their *s*-triazine degradation potential are required. Diverse microorganisms able to degrade these herbicides have been isolated (Mandelbaum *et al.*, 1995; Struthers *et al*., 1998; Topp *et al*., 2000a, 2000b; Hernández *et al*., 2008a; Hernández *et al*., 2008b). *Pseudomonas* sp. ADP has become the model strain for *s*-triazine biodegradation studies. This bacterial strain is able to use atrazine as the sole nitrogen source and its atrazine catabolic pathway has been extensively characterized (Martínez *et al.,* 2001). The enzymes of the upper pathway, which are responsible for the conversion of atrazine to cyanuric acid, are encoded by the *atz*A, *atz*B and *atz*C genes. The lower pathway mineralizes cyanuric acid and is encoded by the *atzDEF* operon (Martínez *et al.,* 2001). The herbicide simazine is also degraded by these catabolic enzymes (Fig. 1).

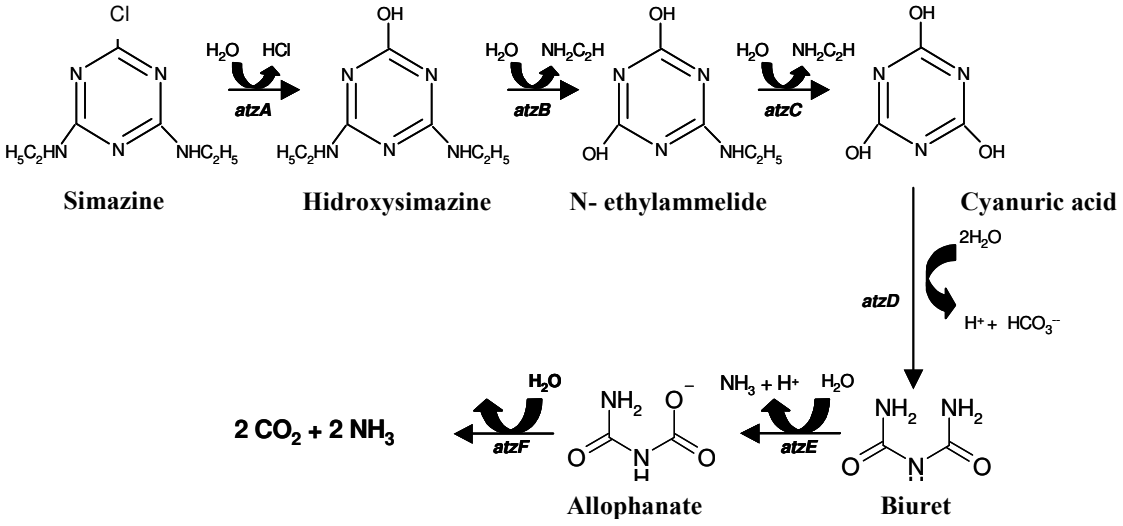

Figure 1: The simazine catabolic pathways. The *atz* genes encoding the metabolic enzymes are indicated.

Figura 1: Vías catabólicas de simazina. Se indican los genes *atz* que codifican las enzimas metabólicas.

In this review, the isolation and characterization of *s*-triazine-degrading bacteria were reported. Studies on *s*-triazine retention and bioavailability in soil were presented. Modern methods for the analysis of microbial *s*-triazine catabolic activities and catabolic genes of soils were presented as a powerful combination of culture-dependent and culture-independent techniques for laboratory, microcosm and field-scale studies. Finally, microbial ecology methods for the study of the structure and the dynamics of soil bacterial communities were reviewed.

Bacterial degradation of *s*-triazines

Bioremediation employs microorganisms to degrade pollutants in the environment. Gram- negative and Gram-positive bacteria, predominantly *Pseudomonas*, *Arthrobacter*, *Pseudaminobacter* and *Nocardiodes* strains, capable to degrade *s*-triazine herbicides have been described (Yanze-Kontchou and Gschwind, 1994; Mandelbaum *et al.*, 1995; Rousseaux *et al*., 2001; Topp *et al.,* 2001; Strong *et al.*, 2002).

To isolate microorganisms able to degrade *s*-triazines, we sampled agricultural soils from avocado and persimmon tree plantations annually treated with simazine in central Chile (Hernández *et al*., 2008b). Isolation was made by enrichment procedures in minimal medium using simazine as sole nitrogen source (Hernández *et al*., 2008a). Diverse strains able to degrade and use simazine as the sole nitrogen source for gowth were isolated from these soils. Some of these bacteria were characterized by sequencing 16S rRNA genes and identified as species of *Pseudomonas*, *Rodococcus*, *Stenotrophomonas* and *Arthrobacter*. The simazine degradation potential of these bacterial strains was studied by resting cells assays. A novel *s*-triazine-degrading *Pseudomonas* strain was further characterized (Hernández *et al*., 2008a). These microorganisms are potential biocatalysts for bioremediation processes to remove *s*-triazines from polluted environments.

Soil characterization

Soil properties and *s*-triazine retention

Several factors determine the persistence of herbicides in soil (Novak, 1999). Soil physical and chemical properties such as texture, organic matter content as well as the pH strongly influence herbicides persistence. In addition, chemical properties of the herbicide

such as solubility, vapor pressure, and the susceptibility to chemical or microbial degradation determine the environmental stability of the compound. Climatic variables, mainly moisture and temperature, are also relevant factors. However, microbial abundance and metabolic activity of soil microorganisms play a central role on herbicide fate in soil (Newcombe and Crowley, 1999).

Retention and mobility of a pesticide in soil are determined by sorption processes, which are governed by physical-chemical properties of the soils and the pesticides (Spark and Swift, 2002). Sorption interactions of pesticides in the soil matrix may involve the mineral and/or organic components (Li *et al.*, 2003). For soils with high organic matter levels (>5%), pesticide retention has been associated with the binding to organic matter (Jenks *et al.*, 1998). In soils with low organic matter content, pesticide adsorption depends on active components of the inorganic fraction, predominantly the clay. It has been postulated that an increase in the clay content decreases the mobility of the pesticide (Cox *et al.*, 2000). Adsorption isotherms are commonly used to determine the herbicide affinity to soil and are often described by Freundlich- or Langmuir-type models (Calvet *et al.*, 1989). Some studies describe the retention of *s*-triazines herbicides by soil constituents. Recently, simazine adsorption behavior was studied in agricultural soils of Aconcagua valley, central Chile (Flores *et al.,* 2008). Soil organic matter and clay minerals are main sorbents for *s*-triazines (García-Valcárcel and Tadeo, 1999; Flores *et al.,* 2008).

In conclusion, physicochemical characteristics of the herbicide, the active surface of the minerals and the organic content of soils as well as the amount of herbicide applied are important parameters for understanding the dynamics of *s*-triazine herbicides in the environment. To protect surface- and groundwater from pesticide contamination, and to ameliorate their impact, broad knowledge is required concerning their sorption-desorption processes in the environment.

Microbiological and molecular analysis of soils

To establish bioremediation processes that remove s-triazines, the detection of active indigenous microbial communities able to degrade these herbicides is required. Recently, an interesting method for the detection and enumeration of MPN of *s*-triazine-degrading microorganisms in soil has been described (Dinamarca *et al.,* 2007). This method

is based on the ability of bacteria to use a *s*-triazine as sole nitrogen source. The metabolic activity of microorganisms is detected by the reduction of 2,3,5-triphenyl-2H-tetrazolium chloride into a colored formazan product. The simazine-degrading activities of microorganisms in an avocado plantation soil from central Chile are shown in Figure 2. The microbial catabolic activities in soils can be attributed to the previous history of simazine treatment (Rousseaux *et al*., 2001; Ralebitso *et al*., 2002, Morán *et al.,* 2006).

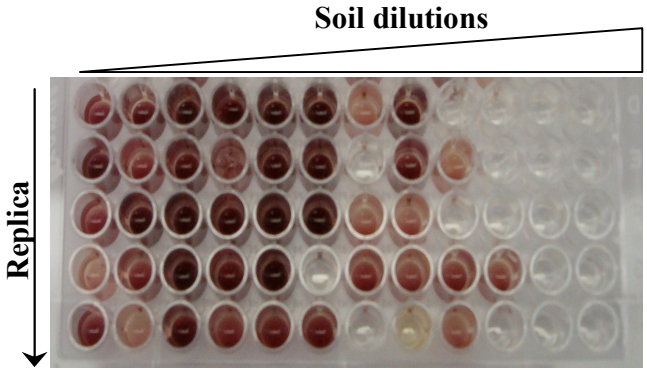

Figure 2. Detection of *s*-triazine degrading activities in soils by MPN-TTC method. Ten-fold serial dilutions of soil sample were prepared and 5 wells of a microtiter plate were inoculated. At the end of incubation, TTC was added (0.01% final concentration) and the microtiter plate was incubated at 30ºC during 4 h. A positive reaction is determined by visualization of the color change by the production of formazan (red color).

Figura 2. Detección de actividad degradadora de *s*-triazina en suelos mediante el método NMP-TTC. Se prepararon diluciones seriadas (1/10) de muestras de suelo y se inocularon en cinco pocillos de una microplaca. Al final de la incubación, se adicionó TTC (concentración final de 0,01%) y se incubó la microplaca a 30ºC durante 4 horas. El cambio de color por la producción de formazán (color rojo) indica una reacción positiva.

The relative abundances of *s*-triazine catabolic genes in soil can be analyzed by a MPN-PCR technique. The detection of *atzA* gene in DNA extracted directly from this soil is illustrated in Figure 3.

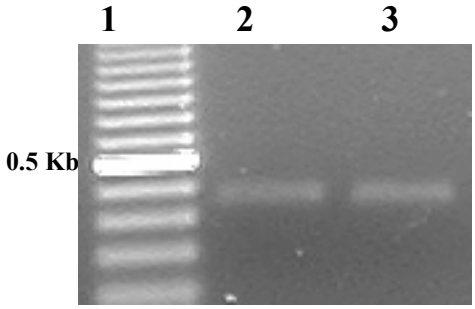

Figure 3. Amplification of catabolic *atzA* gene from soils treated with simazine. Molecular mass markers (line 1), freshly applied herbicide soil (line 2), soil four weeks after herbicide application (line 3).

Figura 3. Amplificación del gen catabólico *atzA* desde muestras de suelos tratados con simazina. Marcadores de masa molecular (línea 1), suelo recién tratado con el herbicida (línea 2), suelo tratado con el herbicida hace 4 semanas (línea 3).

The PCR-amplification of *atzA* in agricultural soils was performed according to the protocols and primers previously described (de Souza *et al*., 1998). The presence of the *atzA* gene in soils is in accordance with the detection of catabolic activities of indigenous simazine-degrading microorganisms. The *s*-triazine catabolic activities quantified by the MPN-TTC method and the catabolic genes determined by the MPN-PCR technique correlates with simazine removal from these soils.

Bioremediation studies

Bioremediation is an effective technology for the clean-up of polluted environments. This biotechnology has several advantages compared to the physicochemical treatments: lower operational costs, *in situ* application, permanent elimination of the residue, and minimum disturbance of the treated site (Ralebisto *et al.,* 2002; Philp and Atlas, 2005; Navia and Seeger, 2006). The most efficient methods for transforming a contaminant into a less-harmful end product are biostimulation and bioaugmentation. Biostimulation involves treating the contaminated soils to increase the pollutant bioavailability, or adding a nutritional supplement or co-substrate to increase the population of contaminant-degrading indigenous bacteria (McTavish, 2001). Bioaugmentation

involves the inoculation of contaminated soils or water with specific microbial strains or consortia to improve the biodegradation capacity of the system for a specific organic pollutant (Philp and Atlas, 2005).

Successful bioaugmentation of *s*-triazine-polluted soils has been described (Yanze-Kontchou and Gschwind, 1994, Alvey and Crowley, 1996, Newcombe and Crowley, 1999, Morgante *et al.,* 2008). For bioaugmentation strategies, bacterial strain selection, inoculum size and inoculation system are important parameters. Bacterial strain selection is critical for bioremediation strategies. The strains used for bioremediation should posses exceptional degrading capacities and important growth rates (Philp and Atlas, 2005; Navia and Seeger, 2006). Therefore, the adaptability of the inoculated strain to the soil and the long term survival of the microorganism are desirable properties. Higher bioremediation efficiency was obtained by inoculating native strains (Rousseaux *et al.,* 2002). Secondly, to increase the biodegradation rate, the native strain has to be inoculated to the polluted site at high densities. For soil bioremediation a higher inoculum size is required than for bioaugmentation of aquatic ecosystems. A high inoculum size allows overcoming competition with native bacteria, predation by protozoa and bacteriophage and lower pollutant bioavailability due to sorption mechanisms in soil (Philp and Atlas, 2005). For atrazine bioremediation in soils, different inoculum sizes have been tested (Topp, 2001; Rousseaux *et al.,* 2002). Finally, the inoculation system is an essential factor in bioaugmentation. Inoculation of cells immobilized in alginate matrices, or other polymeric materials, is a strategy of increasing importance. For example, alginate allows passage of nutrients and excretion products, protects bacteria from predators and nutrient stress and preserves viability of the organisms (Newcombe and Crowley, 1999). Bioaugmentation with repeated inoculations could be useful for increasing bioremediation efficiency. Repeated applications of the strain overcome long-term survival problems (Newcombe and Crowley, 1999). Recently, a successful bioaugmentation strategy for agricultural soil using the native simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 was reported (Morgante *et al.,* 2008). After bioaugmentation with strain MHP41, simazine catabolic activities were increased and herbicide removal was enhanced.

Analysis of soil microbial communities by molecular methods

Microorganisms play key functions in soil and are able to adapt to changing environmental conditions. Thus, variations in bacterial populations and activities may serve as excellent indicators of changes in soil health (Torsvik and Overas, 2002). Most microbes in environmental samples cannot be cultured in laboratory media, which are biased for the growth of specific microorganisms (Torsvik *et al.,* 2002). Molecular biology techniques and microbial culture-independent approaches are increasingly employed for the study of the microbial ecology in complex environments (Alfreider *et al.*, 1996; Muyzer and Smalla, 1998; Osborn, *et al.,* 2000; Nogales *et al*., 2001). The 16S rRNA gene sequence is important for the analysis of microbial diversity and is a relevant marker for studying the phylogeny of bacteria (Fig. 4).

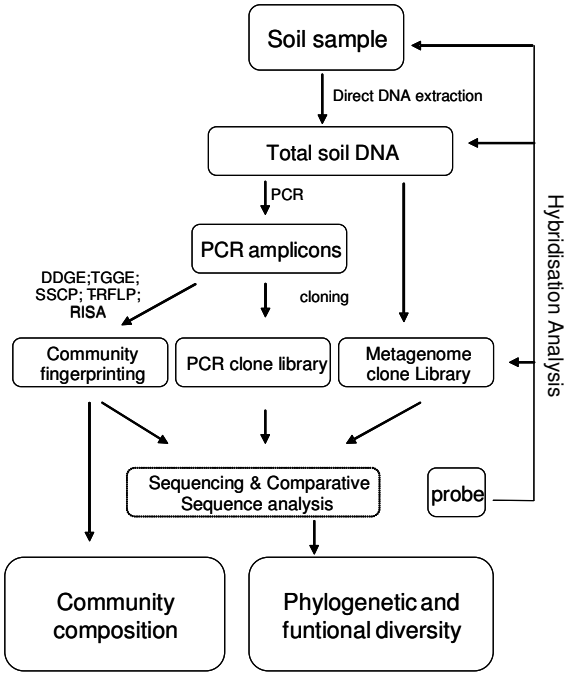

Figure 4. Culture-independent molecular methods for the analysis of microbial communities. PCR: polymerase chain reaction; DGGE: denaturant gradient gel electrophoresis; TGGE: temperature gradient gel electrophoresis; SSCP single strand conformation polymorphism; T-RFLP: terminal restriction fragment length polymorphism; RISA: ribosomal intergenic spacer analysis.

Figura 4. Métodos moleculares cultivo independientes para el análisis de comunidades microbianas. PCR: reacción en cadena de la polimerasa; DGGE: electroforesis en gel con

gradiente desnaturalizante; TGGE: electroforesis en gel con gradiente de temperatura; SSCP: polimorfismo de la conformación de cadena simple de ADN; T-RFLP: polimorfismo de longitud de fragmentos de restricción terminales; RISA: análisis del espaciador intergénico ribosomal.

To date, most studies quantify the depletion of the contaminant or monitor cultivable microorganisms or their catabolic activities in polluted soils. Only few reports describe the dynamics of microbial communities throughout the biodegradation process (Piutti *et al.*, 2003; Moreno *et al*., 2007). The knowledge of the microbial communities inhabiting *s*-triazine contaminated soils and their response to bioaugmentation strategies is useful for the identification of the microorganisms that are adapted to these compounds and are involved in their degradation. Changes in the bacterial community structure in soil by pesticides application have been observed (Engelen *et al*., 1998; El Fantroussi *et al.*, 1999). The structure of the microbial community in an atrazine-contaminated soil changed in response to soil organic amendments (Martin-Laurent *et al*., 2004) and carbon and nitrogen source availability (Rhine *et al*., 2003). Microbial community structure of *s*-triazine treated soil has been studied by FISH (Barra Caracciolo *et al.*, 2005). Specific groups of bacteria such as α-, β- and γ-subdivisions of *Proteobacteria*, Gram-positive bacteria of high GC DNA content and *Planctomycetes* were detected. The presence of *s*-triazines affected the bacterial community structure.

The bacterial community structure of a pesticide-contaminated site and the changes induced in the community structure during bioremediation approaches has been recently described (Paul *et al*., 2006). The microbial community of this pesticide-contaminated soil was mainly constituted by *Proteobacteria* and *Actinomycetes*. Bioaugmentation enhanced pollutant degradation. However, T-RFLP analysis revealed non-significant changes in bacterial community structure during the bioremediation process. In Chilean agricultural soils, we evaluated the capability of native soil microbiota and bioaugmentation strategies on simazine biodegradation by microcosm experiments. The 16S rRNA gene pool amplified from the soil genomic library was cloned and FISH and T-RFLP was performed to analyze changes in soil microbial community structure due to simazine amendment and bioaugmentation strategies. Our results showed that the native soil microbiota was able to

degrade simazine. However, the addition of a native strain enhanced simazine-degrading activities and was essential for increasing the attenuation of simazine in soil. Sequencing of representative clones of soil bacteria showed that the microbial community structure was mainly constituted by *Proteobacteria*, *Actinomycetes*, *Acidobacteria* and *Planctomycetes*. Microbial community analysis by T-RFLP revealed that simazine application and bioaugmentation promotes changes in the structure of soil microbial communities, while FISH indicates variations in some specifics bacterial groups.

Conclusions

In this review, the basis of bioremediation of herbicides in agricultural soils was analyzed. Diverse simazine-degrading strains isolated from Chilean agricultural soils were characterized. The MPN-TTC method was used for the estimation of the *s*-triazine-degrading activities in soil. Catabolic genes for *s*-triazine degradation were detected in soil by MPN-PCR. Bacterial community adapted to the herbicide application and with simazine catabolic capabilities are present in Chilean agricultural soils. Culture-independent molecular methods such as FISH, T-RFLP and clone libraries were used to understand the effects of herbicide application and bioaugmentation on soil microbial communities. The isolated *s*-triazine-degrading microorganisms are novel biocatalysts that were used for the development of bioaugmentation strategies.

Acknowledgments

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Chapter 3

Part I

Novel *s*-triazine-degrading bacteria isolated from agricultural soils of central Chile for herbicide bioremediation

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Abstract

s-Triazine-degrading bacterial strains were isolated from long-term simazine-treated agricultural soils of central Chile. The total heterotrophic bacteria of these agricultural soils (7 x 10^6 CFU/g of dry soil) were not affected after simazine application on field. By enrichment in minimal medium containing simazine as the sole nitrogen source, the bacterial strains P51, P52 and C53 were isolated and selected based on their simazine-degrading abilities. Resting cells of strains P51 and P52 degraded >80% of simazine within 48 h, whereas strain C53 was able to remove >60% of the herbicide. The *atzA* and *atzD* genes of the *s*-triazine catabolic pathways were detected in strains P51 and C53, while only *atzD* gene was observed in strain P52. To compare the bacterial 16S rRNA gene restriction pattern, ARDRA were performed using the restriction enzymes *Msp1* and *Hha1*. ARDRA indicate that strain P52 is a different ribotype that C53 and P51 strains. For further characterization, the novel isolates were identified by 16S rRNA gene sequencing. Strains C53 and P51 belong to the genus *Stenotrophomonas* and the strain P52 belongs to the genus *Arthrobacter*. *s*-Triazine-degrading bacteria isolated from contaminated soils could be used as biocatalysts for bioremediation of these herbicides.

Introduction

Soil decontamination is one of the main environmental challenges for the third millennium to safeguard the planet. The herbicides are the most abundant agrochemicals used worldwide and their application has increased in the last decades. The United States is the main herbicide consumer worldwide, with an annual application of around 205,000 tons (Environmental Protection Agency, 2004. Pesticides industry sales and usage, EPA, Washington, DC, USA). The European Union registered an annual consumption of around 130,000 tons of herbicides between 1990 and 2000 (Food and Agriculture Organization of the United Nations, 2006. Statistical database, FAO, Rome, Italy), and France (26%), United Kingdom (17%) and Italy (12%) head the herbicide usage. In South America, the annual use of herbicides was around 60,000 tons between 1990 and 2000 (Figure 1). Brazil (38%), Argentina (28%) and Colombia (18%) are the main herbicide consumers in South America.

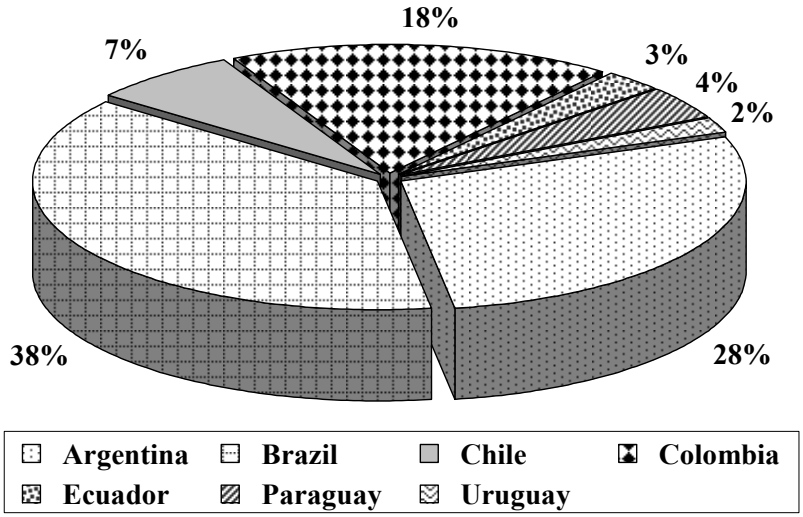
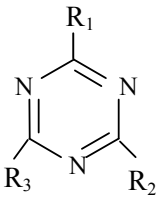


Figure 1. South America's herbicide consumption from 1990 to 2001. Food and Agriculture Organization of the United Nations, 2006. Statistical database, FAO, Rome, Italy.

s-Triazine compounds have been used as weed control in many countries for more than 40 years (Wackett *et al.,* 2002; Dinamarca *et al.,* 2007). These synthetic and chlorinated herbicides (Figure 2) inhibit the electron transfer during the photosynthesis in target plants (Rousseaux *et al.,* 2001). For agriculture, *s*-triazine main applications are as a pre- and post-emergence herbicides in crop fields, controlling broadleaf and grassy weeds in corn fields, citrus crops, alfalfa, grapes, avocado and olives (Wackett *et al.,* 2002; Cooman *et al.,* 2005). Their non-agricultural uses include weed control on right-of-ways, ornamental trees and golf fairways. In 2001, the European Union reported a list of "priority hazardous substances" (European Parliament and the Council of the European Union, 2001. Decision 2455/2001/EC, Brussels, Belgium). Among these products, atrazine and simazine have been included due to their persistence, toxicity, moderate leaching capacity and potential to adsorb onto soils and sediments. Commonly, levels of *s*-triazine herbicides detected in water exceed the maximum contaminant levels allowed for drinking water in European Union (0.1 µg/l) and USA (3.0 µg/l) (Rousseaux *et al.,* 2001). Although *s*-triazines have been prohibited in many European countries, in Chile atrazine and simazine are still the most frequently applied herbicides for weed control in agriculture and forestry practices, and there are no restrictions for their use. *s*-Triazines consumption in Chile was

around 350 tons during 2004 (Dinamarca *et al.,* 2007), which represents 10% of the herbicide usage. *s*-Triazine herbicides have been recently detected as contaminants in agricultural watersheds in south-central Chile (Cooman *et al.,* 2005).



***s*-Triazine**	**R_1**	**R_2**	**R_3**
Atrazine	Cl	NHC_2H_5	$NHCH(CH_3)_2$
Cianazine	Cl	$NHC(CN)(CH_3)_2$	$NHCH(CH_3)_2$
Propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
Simazine	Cl	NHC_2H_5	NHC_2H_5

Figure 2. Chemical structures of *s*-triazines. Members of *s*-triazine herbicides differ by their R_1, R_2 y R_3 groups around the *s*-triazine ring.

Degradation by microorganisms is the primary removal mechanism of *s*-triazine herbicides from the soil (Newcombe and Crowley, 1999; Dinamarca *et al.,* 2007; Hernández *et al.,* 2008a). For environmental bioremediation, the isolation of microorganisms capable of degrading the target pollutant is important. A number of *s*-triazine-degrading bacteria have been described (Mandelbaum *et al.,* 1995; Devers *et al.,* 2007; Hernández *et al.,* 2008b). The bacterial enzymes for *s*-triazine degradation are encoded by *atzA, atzB, atzC, atzD, atzE* and *atzF* genes (Figure 3) (de Souza *et al.,* 1998; Martínez *et al.,* 2001; Iwasaky *et al.,* 2007).

The aim of this study was to isolate novel bacterial strains capable to degrade *s*-triazines from agricultural soils in the Quillota valley, central Chile. For enrichment and isolation of bacterial strains, long-term simazine treated soils were used. Physiological, biochemical and molecular studies were carried out to characterize and identify bacterial isolates, which could be used as novel biocatalysts for soil bioremediation.

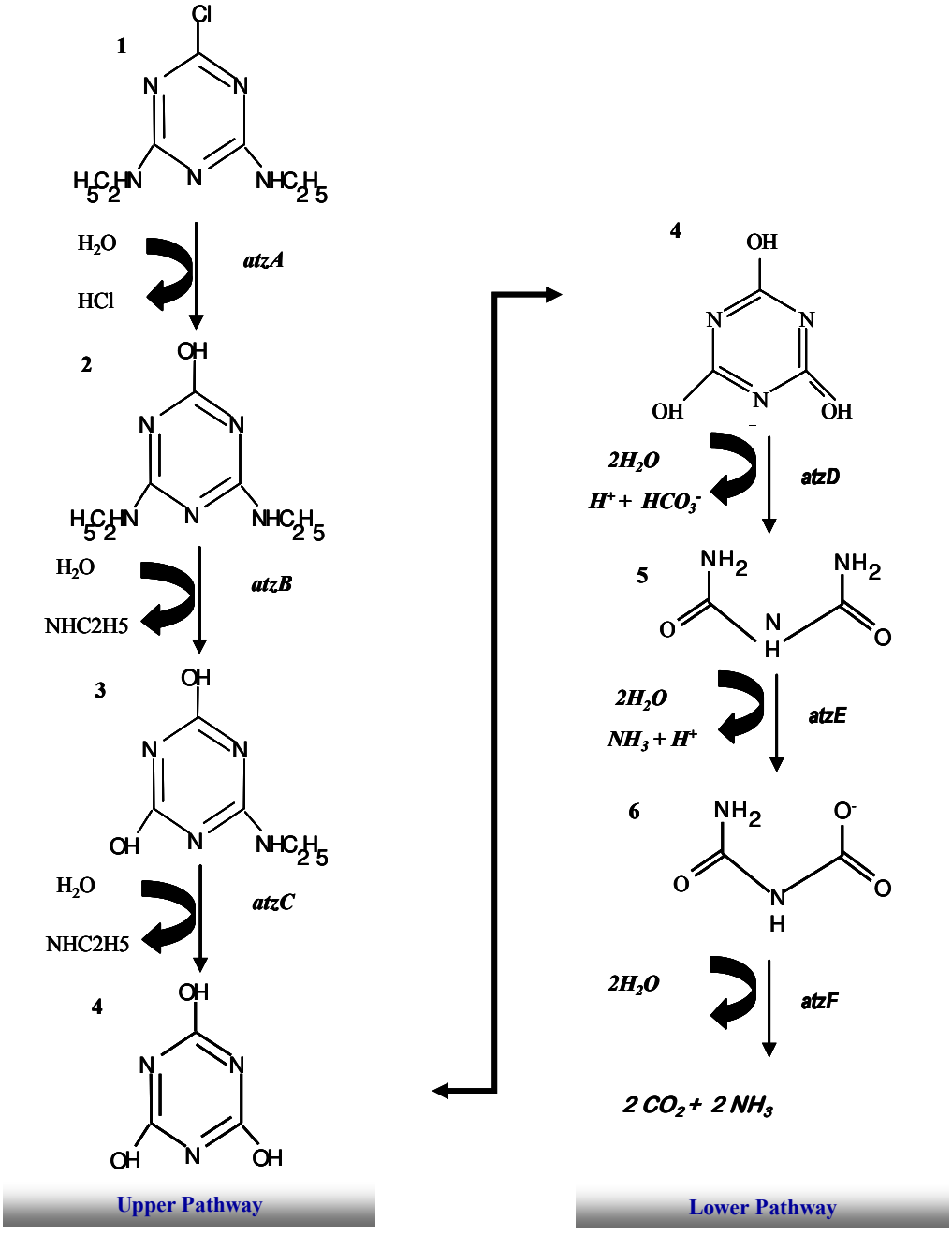


Figure 3. The simazine catabolic pathways. The catabolic *atz* gene encoding the respective enzyme is indicated at each metabolic step. Pathways substrates and metabolites: 1. Simazine; 2. Hydroxysimazine; 3. N-etilammelide; 4. Cyanuric acid; 5. Biuret; 6. Allophanate.

Material and methods

Materials

Commercial simazine (Gesatop WP90) was purchased from Syngenta (Greensboro, United States). Simazine (99% pure) and standard simazine (>99% pure) were obtained from Atanor (Buenos Aires, Argentina) and Dr. Ehrensdorfer-Schäfers GmbH (Augsburg, Germany), respectively.

Soil sampling

Soil samples were obtained from agricultural sites treated with herbicide simazine in Quillota valley, central Chile. The sampling sites correspond to an avocado (*Persea americana*) and a persimmon (*Diospyros chinensis*) plantations that have been annually treated with commercial simazine for more than 20 years, according to farming practices for the control of annual weeds. Soil samples were collected in December 2002 from the first soil stratum (0-20 cm). Samples were sieved, air dried and stored at 4 ºC.

Isolation of herbicide-degrading bacteria

The total heterotrophic bacteria were estimated by plating on tryptic soy agar (TSA) plates. The isolation of native simazine-degrading bacterial strains was performed by batch enrichment cultivation. 10 g of soil (dry weight basis) was inoculated in 90 ml of atrazine medium (AM) (Rousseaux *et al.,* 2001), modified using commercial simazine (Gesatop WP90) as sole nitrogen source. Cycloheximide (50 mg/l) was added to the medium to inhibit eukaryotic cell growth. Cultures were incubated at 28 ºC with orbital agitation. Simazine was incorporated into the AM agar to a final concentration of 2.5 mM (Rousseaux *et al.,* 2001). The AM agar cultures were incubated at 28 ºC. Colonies on AM agar were purified and maintained on this medium. Strains were grown using simazine as sole nitrogen source. Strains were stored at -24 ºC with 20% glycerol.

Physiological and biochemical characterization

Bacterial characterization included physiological and biochemical analyses of strains able to grow using simazine as sole nitrogen source. For this propose, the strains were cultured in AM broth containing simazine 2.5 mM. The characterization of the newly

selected isolates was determined by conventional methods: Gram staining, catalase and oxidase activities and motility. The morphology of bacterial colonies on TSA plates was observed. The cell growth in LB broth (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) supplemented with simazine (2.5 mM) was determined by measuring turbidity at 600 nm. For electron microscopy, bacterial cells were treated and observed with a Zeiss EM900 electron microscope as described previously (Cámara *et al.,* 2004).

Analysis of simazine degradation and catabolic genes

Bacterial cultures were grown in LB broth supplemented with simazine at 30 ºC to exponential phase. Cells were washed and resuspended to a turbidity $_{600\ nm}$ ~5.0 using 10 mM sodium phosphate (pH 7.0). Resting cells of each strain were incubated with simazine (0.5 mM) until 48 h. Simazine was extracted with isooctane and measured by high-performance liquid chromatography (HPLC). The samples were analysed, using a Beckman liquid chromatography equipped with a diode array detector with a RP-C18/Lichrospher 5-µm column (Supelco, Bellefonte, USA) (Martínez *et al.,* 2007). The samples were eluted at a flow rate of 0.5 ml/min using a mobile phase containing 67% (v/v) acetonitrile, 32.5% (v/v) water and 0.5% (v/v) phosphoric acid (pH 2.0). Simazine was identified and quantified by comparison with an authentic standard.

For the analyses of catabolic genes, the *atzA* and *atzD* genes were amplified by PCR from genomic DNA using specific primers (de Souza *et al.,* 1998; Devers *et al.,* 2004).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Genomic DNA was isolated from a single colony grown over night in LB broth by using Wizard genomic kit (Promega, Madison, WI, USA). 16S rRNA genes were amplified,using primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification were carried out with the following conditions: 1 cycle of 94 ºC for 3 min, 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 45 s, plus a final extension at 72 ºC for 7 min. The PCR products were digested with the endonucleases *MspI* and *HhaI* (Promega, Madison, WI, USA), according to the protocols of the manufacturer. ARDRA profiles (5 µl of digested 16S rRNA gene products) were

visualized after electrophoresis in an agarose (3% w/v) gel in TBE buffer. The gel was stained with ethidium bromide and visualised under UV light.

Identification of bacterial strains by 16S rRNA gene sequence analysis

DNA was extracted using the BIO101 DNA Kit for bacteria (Qiagen, Hilden, Germany). For PCR amplification of 16S rRNA genes, the 27f and 1492r primers were used. Amplification reaction was performed with a DNA Engine PTC-200 Peltier Thermal Cycler (MJ Research, Massachusetts, USA), with the following programme: 1 cycle of 95 ºC for 5 min; 35 cycles of 95 ºC for 45 s; 55 ºC for 45 s; 72 ºC for 2 min; followed by a final extension at 72 ºC for 10 min. Amplification products were visualised by electrophoresis in agarose (1.0% w/v) gel and staining with ethidium bromide. PCR 16S rRNA genes were purified, using QiaQuick columns and the protocol of the manufacturer (Qiagen, Hilden, Germany), and sequenced directly, using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit, version 3.1, an ABI model 3100 DNA Sequencer and the protocols of the manufacturer (Applied Biosystems Inc., Foster City, CA, USA).

Results and discussion

Isolation of *s*-triazine-degrading bacteria

The adaptation to long-term polluted environmental sites endows environmental microbes with the capabilities to deal with these pollutants. Soils treated with the herbicide simazine for more than 20 years were used for isolation of bacterial strains able to degrade *s*-triazines. Microbial communities from these soils were enriched and acclimated in AM broth under aerobic conditions using simazine as sole nitrogen source. The total heterotrophic bacteria from the studied agricultural soils were estimated by plating on TSA medium. Total heterotrophic bacteria counts were 7 x 10^6 CFU/g of dry soil and this number was not affected after simazine application. Simazine-degrading bacterial strains were isolated by enrichment. Soil samples were inoculated in AM broth with simazine as the sole nitrogen source. Colonies growing on AM agar with simazine as sole nitrogen source were isolated. From cultivable microbes of agricultural soils, three bacterial isolates capable of simazine-degradation were selected and characterized. Strains P51 and P52 were enriched from an avocado plantation soil, and strain C53 was isolated from a persimmon

plantation soil. The bacterial strains were characterized by biochemical and physiological analyses (Table 1). Figure 4 shows the colony morphology on TSA medium and cell morphology observed by transmission electronic microscopy of strains P51 and P52. The three bacterial strains grow in LB broth supplemented with simazine, reaching turbidity $_{600\ nm}$ ~1.2. Degradation of simazine by these strains grown in LB broth with simazine was studied using resting cell assays. The strains P51 and P52 degraded more than 80% of simazine (0.5 mM) within 48 h, while strain C53 removed more than 60% of this herbicide in this assay (Figure 5). The presence of the catabolic *atzA* and *atzD* genes was analyzed by PCR-amplification using specific primers. The *atzA* and *atzD* genes are crucial in *s*-triazine degradation (Mandelbaum *et al.,* 1995; Devers *et al.,* 2007; Iwasaky *et al.,* 2007) and encode the first enzyme of the "upper" and "lower" *s*-triazine catabolic pathways (Figure 3), respectively. The *atzA* and *atzD* genes were detected in strains P51 and C53, while only *atzD* gene was observed in strain P52 (Table 2). The isolation of *s*-triazine-degrading bacteria has been reported, and *atz* genes have been determined in some of these isolates (Mandelbaum *et al.,* 1995; Rousseaux *et al.,* 2001; Devers *et al.,* 2007).

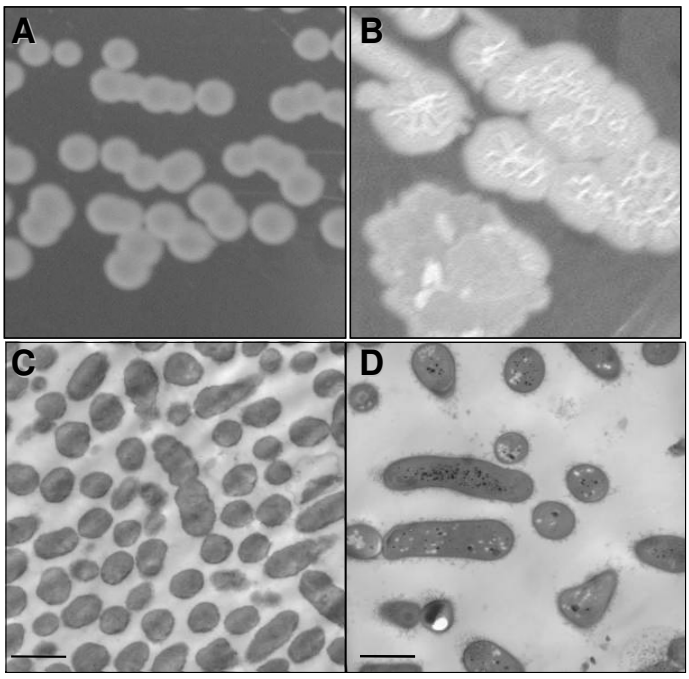


Figure 4. Colony and cell morphology of simazine-degrading bacterial strains.
A) *Stenotrophomonas* sp. strain P51, colony morphology on TSA medium.

B) *Arthrobacter* sp. strain P52, colony morphology on TSA medium.

C) *Stenotrophomonas* sp. strain P51, bacterial cell morphology observed by electronic microscopy.

D) *Arthrobacter* sp. strain P52, bacterial cell morphology observed by electronic microscopy.

Bar represents 1 μm.

Table 1. Biochemical and physiological characterization of the novel bacterial strains isolated from agricultural soil

Strain	Gram	Catalase	Oxidase	Motility
P51	-	+	-	+
P52	+	+	-	-
C53	-	+	-	+

Table 2. Bacterial characterization by 16S rRNA gene sequence analysis and PCR-amplification of atzA and atzD genes.

Strain	Identification (16S rRNA)	Similarity (%)	*atzA* gene	*atzD* gene
P51	*Stenotrophomonas*	>98	+	+
P52	*Arthrobacter*	>97	-	+
C53	*Stenotrophomonas*	>99	+	+

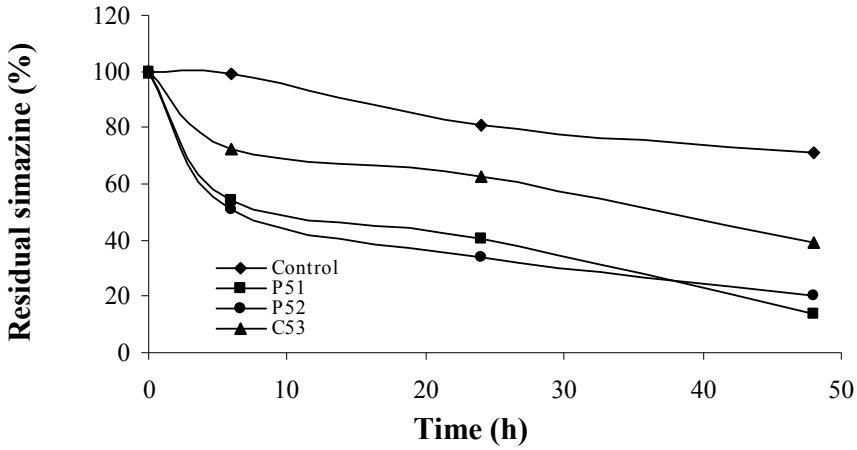


Figure 5. Simazine degradation by the isolates P51, P52 and C53. Resting cells of cultures previously grown in LB broth with simazine were incubated with simazine (0.5 mM). Control: incubation without cells. Each value is an average of two independent experiments.

ARDRA

To further characterize the isolates, comparative analysis of the 16S rRNA gene restriction pattern (ARDRA) of the bacterial strains was performed. This analysis provides a fast analysis of the 16S rRNA gene sequence structure of bacterial organisms for comparison between restriction profiles of target strains. The PCR-amplified 16S rRNA genes of the three strains were digested using the restriction enzymes *Msp1* and *Hha1*. The ARDRA profiles of P51 and C53 strains were identical, suggesting that they are closely related bacteria. ARDRA indicates that strain P52 is a different ribotype (Figure 6).

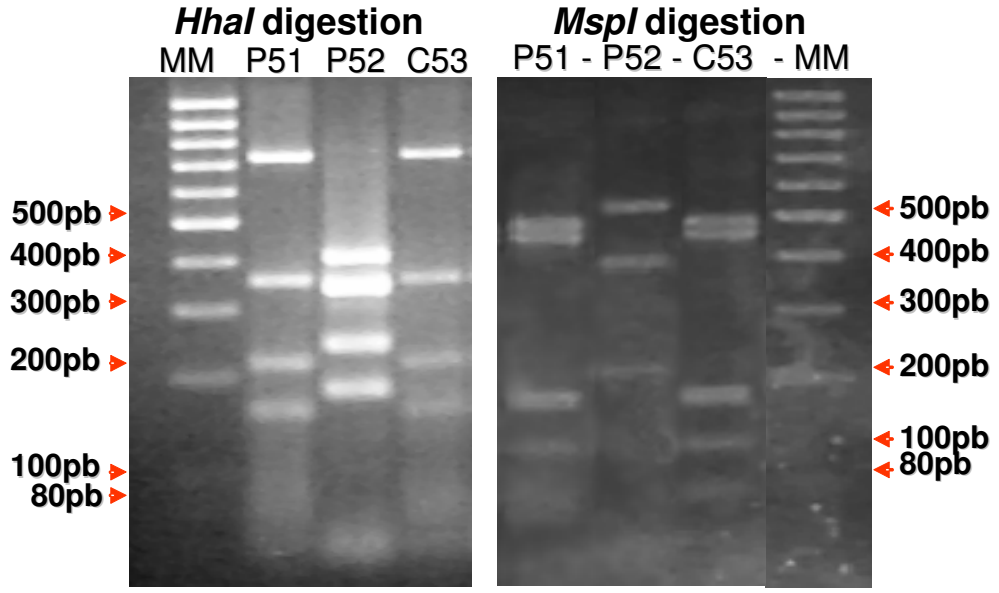


Figure 6. Amplified rRNA gene restriction analysis of 3 isolated bacterial strains. DNA restriction patterns were observed in an agarose gel stained with ethidium bromide. The amplified 16S rRNA genes were digested with restriction enzymes *Hha*I and *Msp*I. MM: Molecular mass standards.

Identification of simazine-degrading bacteria

The 16S rRNA gene of the three isolates were sequenced and analysed for bacterial identification. The strain C53 isolated from persimmon plantation and strain P51 from avocado plantation soil were classified as *Proteobacteria*, class *Gammaproteobacteria*, order *Xanthomonadales*, family *Xanthomonadaceae* and were most similar to genus *Stenotrophomonas*, with a similarity higher than 98%. Both strains differ slightly in their 16S rRNA gene sequence. The strain P52 isolated from persimmon plantation soil was classified as *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, family *Micrococcaceae* and was most similar to genus *Arthrobacter* with a similarity higher than 97% (Table 2). *s*-Triazine-degrading bacterial strains belonging to other genera, such as *Pseudomonas*, *Nocardioides* and *Agrobacterium*, have also been reported (Mandelbaum *et al.,* 1995; Devers *et al.,* 2007; Hernández *et al.,* 2008b).

Conclusions

Three bacterial strains capable of degrading simazine and to grow using it as sole nitrogen source were isolated from herbicide-treated agricultural soils of central Chile. These strains were further characterized and identified. Resting cells of the strains P51, P52 and C53 were able to degrade efficiently simazine. The *atzA* and *atzD* genes encoding the first enzyme of the "upper" and "lower" *s*-triazine catabolic pathways were detected in strains P51 and C53. The bacterial strain P52 showed the presence of *atzD* gene. ARDRA allowed comparing the restriction profiles of the three isolated strains, indicating that strains C53 and P51 have the same restriction pattern. The strain P52 is a different ribotype. By sequence analysis of the 16S rRNA genes, strains C53 and P51 were identified as *Stenotrophomonas* sp. and strain P52 was identified as *Arthrobacter* sp. Further studies of these simazine-degrading bacteria are required to analyze their potential as biocatalysts for bioremediation of environments contaminated with *s*-triazine herbicides.

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Chapter 3

Part II

Molecular characterization and phylogeny of novel herbicide-degrading *Stenotrophomonas* sp. strains isolated from Chilean agricultural soil

Abstract

Simazine has been one of the most extensively used *s*-triazine herbicides for weed control in agricultural soils. Native *s*-triazine-degrading microorganisms have been used for bioremediation of polluted sites. The aim of this study was to characterize strains of *Stenotrophomonas* spp. and their relevance for simazine degradation. Diverse *Stenotrophomonas* strains, isolated by enrichment from two agricultural soils with histories of simazine application and from a control soil of central Chile, were able to grow on simazine as the sole nitrogen source. A Multi-Locus Sequence Analysis (MLSA), using 16S rRNA genes and *gyr*B and *rpo*D house-keeping genes enabled differentiation and species-level identifications for the isolates as a species of *Stenotrohpomonas*. All simazine-degradation genes, *atzA, atzB, atzC, atzD, atzE* and *atzF*, responsible for s-triazine-mineralization, were detected by PCR-amplification and sequence analyses in four strains. Simazine-degradation by resting cells was studied in two strains that posses all *atz* genes, showing the ability to degrade at least 50% of the initial simazine in less than one hour. All other strains of the *Stenotrophomonas* sp. possessed one or more of the *atz* genes and demonstrated varying degrees of simazine-degradation. The 16S rRNA gene sequence as well as *gyrB* and *rpoD* sequence analyses indicate that the isolated strains probably comprise a distinct and novel species of *Stenotrophomonas*. The effectiveness of a MLSA strategy has been demonstrated for reliable identification of bacterial strains isolated from environmental samples. s-Triazine-degrading bacteria isolated from contaminated soils could be applied as biocatalysts for bioremediation of these herbicides.

Introduction

s-Triazine herbicides have been used world-wide for controlling broad-leaf and grassy weeds (Mandelbaum *et al.*, 1995). Due to the importance of agricultural production in Chile, extensive amounts of herbicides, including simazine have been used. These agrochemicals, used as pre- and post-emergent herbicides, are strong inhibitors of photosynthesis (Foth, 1999; Rousseaux *et al.*, 2001). Diverse pesticides, including chlorinated insecticides and *s*-triazine herbicides, are recalcitrant to degradation (Tappe *et al.*, 2002) and persist in the soil (Rousseaux *et al.*, 2001) as well as in aquifers and

groundwater (Topp *et al.*, 2000a; Topp *et al.*, 2000b). Atrazine has been classified by the United States EPA as a class C carcinogen (Ralebitso *et al.*, 2002; Tappe *et al.*, 2002).

Despite the persistence of *s*-triazine in soils, bacterial strains able to degrade these compounds have been isolated (Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995; Struthers *et al.*, 1998; Topp *et al.*, 2000a; Topp *et al.*, 2000b; Rousseaux *et al.*, 2001; Hernández *et al.*, 2008a; Hernández *et al*., 2008b). Biodegradation, catalysed by microorganisms, is probably the best solution for removing *s*-triazine from soils (Wackett and Hershberger, 2001). The enzymes for *s*-triazine catabolism to cyanuric acid are encoded by the *atzA, atzB* and *atzC* genes (de Souza *et al.*, 1995; Mandelbaum *et al.*, 1995; de Souza *et al.*, 1998a; Strong *et al.*, 2002). Cyanuric acid is catabolised further to carbon dioxide and ammonia by enzymes encoded by *atzD, atzE* and *atzF* genes (Strong *et al.*, 2002).

For the identification of bacteria, a variety of genotypic-based methodologies have been described. Sequencing of the 16S ribosomal RNA (16S rRNA) gene is recognised to be an important tool for estimating phylogenetic relationships between bacteria and the database of 16S rRNA gene sequences comprises the largest collection of a phylogenetic marker (Yarza *et al.*, 2008). The features of this molecular target are useful as a phylogenetic tool and for identifying new environmental bacterial strains. However, the conserved nature and limited resolution of the 16S rRNA gene sequence does not allow the differentiation of closely related species (Clarridge, 2004). The identification of isolates from a variety of taxa have been assessed by comparative nucleotide sequence analyses of less-conserved "housekeeping" genes of the core bacterial genome (Konstantinidis and Tiedje, 2005), such as the genes for DNA gyrase β-subunit (*gyrB*) (Coenye *et al.*, 2004), RNA polymerase D (*rpoD*) (Lonetto *et al.*, 1992), as well as the 16S-23S rRNA inter-genic spacer (IGS-1) region (Guasp *et al.*, 2000).

Stenotrophomonas species are Gram-negative, non-fermenting obligately aerobic bacilli commonly isolated in environmental samples and biotechnological capabilities have been reported for many strains (Coenye *et al.*, 2004). The *Stenotrophomonas* genus consists of eight species, as well as, at least, ten genomovars (Coenye *et al.*, 2004).

The objectives of the present study were to isolate, identify and characterize new simazine-degrading *Stenotrophomonas* isolates from soils in Aconcagua valley , assessing

the applicability of the genes for gyrase β-subunit (*gyrB)* and the RNA polymerase sigma factor (*rpoD*) as biomarkers for the genotypic differentiation and species-level identification of the *Stenotrophomonas* isolates.

Materials and methods

Materials

Commercial simazine (Gesatop WP90) was purchased from Syngenta (Greensboro, United States). Standard simazine (99% purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Sampling sites

Samples were collected from agriculture soils of avocado and persimmon crops with long histories of simazine application, and from a "control" soil without history of simazine application, from the Aconcagua valley, central Chile. The soil samples were labelled "P" (Palta = avocado), "C" (Caqui = persimmon) and "K" (control) and stored at 4ºC. The particle size of the three soil samples were the following: 10.76% of clay, 40.65% of silt and 48.59% of sand for avocado soils; 15.47% of clay, 51.56% of silt and 32.96% of sand for persimmon soils and 17.80% of clay, 56.25% of silt and 25.93% of sand control soils.

Growth media and isolation

Soil samples (10 g, on dry weight basis) were incubated in 90 ml of minimal medium (Hernández *et al.*, 2008a), using simazine as the sole nitrogen source. Cultures were incubated at 28ºC, with shaking at 150 rpm. All enrichment cultures were plated onto a solid medium consisting of AM supplemented with 15-g l^{-1} agar and simazine to a final concentration of 2.5 mM. Colonies on agar medium were purified and maintained on the same medium. Strains were kept frozen at –24ºC with 20% glycerol. All isolates were deposited in the Culture Collection, University of Gothenburg (CCUG).

Identification of bacterial isolates

Bacterial isolates were obtained from the enrichment cultures. Colonies of bacterial isolates were scraped from agar medium and suspended in 100 µl sterile, deionised H_20.

Chromosomal DNA was extracted, using the BIO101 DNA Kit for Bacteria (Q-Biogene, Nottingham, U.K.). Briefly, cells were lysed, by beating with glass beads. DNA was bound to an affinity matrix, washed and eluted. PCR-amplification was carried out, targeting the 16S rRNA genes, using primers M16F27 and M23R458 (Table 1), hybridising at 16S rDNA nucleotide positions 8-27 and 23S rDNA nucleotide positions 458-476 (*Escherichia coli* rRNA gene sequence numbering), respectively.

To determine the differentiation of strains and species identification, comparative analyses of selected regions from DNA gyrase subunit B (*gyrB*) and RNA polymerase subunit D (*rpoD*) "housekeeping" genes of the *Stenotrophomonas* sp. strains were carried out. Housekeeping genes were amplified by PCR using the following primers: U-gyrB-F and U-gyrB-R (external primers), Smal_gyrB-Seq-Fa and Smal_gyrB-Seq-R for gyrB (internal primers) (Table 1) and U-rpoD-F and U-rpoD-R (external primers), Smal_rpoD-Seq-F and Smal_rpoD-Seq-R for rpoD (internal primers) (Table 1).

For the PCR protocols, PCR mixture contained: 5.0 µl of 10X *Taq* polymerase Q-buffer (Qiagen, Crawley, U.K.), 1 µl 10 mM dNTP's (Bioline,U.K.), 2.5 mM $MgCl_2$, 0.8 µg/µl BSA (Roche, Basel, Switzerland), 1.0 µl (50 pmol) of each primer (Operon, Cologne, Germany), 10 µl Q-solution, 1.25 U *Taq* DNA polymerase (Qiagen) and 1.0 µl DNA template, in a final volume of 50 µl. Amplification reactions were performed with a DNA Engine PTC-200 Peltier Thermal Cycler (MJ Research, Massachusetts, USA). Amplification products were visualised by eletrophoresis in a 1.0% agarose gel (Sigma) and staining with ethidium bromide.

PCR products were purified, using QiaQuick columns and the protocol of the manufacturer (Qiagen), and sequenced directly, using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit, version 3.1, an ABI model 3100 DNA Sequencer and the protocols of the manufacturer (Applied Biosystems, Inc., Foster City, California). Sequence data were checked manually and the sequences were submitted to the EMBL Nucleotide Sequence Database (Kulikova *et al.*, 2007), using the FastA3 algorithm (http://www.ebi.ac.uk/fasta3/) (Pearson and Lipman, 1988), and compared to reference 16S rRNA gene sequence data, including those of the type strains of species with validly published names. Sequences were aligned and similarities were calculated, using the multiple alignment tool in the Kodon Bioinformatics package, version 3.0 (Applied Maths, BVBA, Sint-Martens-Latem,

Belgium). Final alignments were completed by hand, using conserved sequence positions and secondary structure as references.

Table 1. Primer sets used in the present study

Primer	Sequence (5'−3')	Reference
atzA-F	CCATGTGAACCAGATCCT	(de Souza *et al.*, 1998a)
atzA-R	TGAAGCGTCCACATTACC	
atzB-F	TCACCGGGGATGTCGCGGGC	
atzB-R	CTCTCCCGCATGGCATCGGG	
atzC-F	GCTCACATGCAGGTACTCCA	
atzC-R	GTACCATATCACCGTTTGCCA	
atzD-F	ACGCTCAGATAACGGAGA	(Fruchey *et al.*, 2003)
atzD-R	TGTCGGAGTCACTTAGCA	
atzE-F	GGTATCGCCTCTGGCAGAAC	(Cheng *et al.*, 2005)
atzE-R	GGCGATACCGGTGTCTTGT	
atzF-F	AAGATCTGGTCGAGTCAC	
atzF-R	TATTGAGCCGCGAGGTATGC	
rpoD external primers		
U-rpoD-F	YATGMGNGARATGGGNACNGT	(Yamamoto *et al*., 2000)
U-rpoD-R	NGCYTCNACCATYTCYTTYTT	
gyrB external primers		
U-gyrB-F	CAYGSNGGNGGNAARTTYRA	(Yamamoto and Harayama, 1998)
U-gyrB-R	GCNGGRTCYTTYTCYTGRCA	
rpoD internal primers		
SmaI_rpoD-Seq-F	NCCSRCCGGTCCGGAYCCG	(Svensson *et al.,* 2010)
SmaI_rpoD-Seq-R	CGTGTCGGTCAGTGGCAGCGG	
gyrB internal primers		
SmaI_gyrB-Seq-Fa	SAGYTTCGTSGARCAYCTGGC	(Svensson *et al.*, 2010)
SmaI_gyrB-Seq-R	TGGCCTGCTTGGCGATGCCG	
16S rRNA primers		
16F27	AGAGTTTGATCCTGGCTCAG	(Hauben *et al.*, 1997)
M23R458	CCCCTTTCCCTCACGGTAC	(Guasp *et al*., 2000)

Amplification and sequence analyses of of *atzA, atzB, atzC, atzD, atzE* and *atzF*

DNA was extracted from all strains as described previously (Hernández *et al.*, 2008a) and the *atzA, atzB, atzC, atzD, atzE and atzF* genes were targeted for amplification by PCR, using primers reported before (De Souza *et al.*, 1998b; Fruchey *et al.*, 2003; Cheng *et al.*, 2005) (Table 1).

Simazine degradation by resting-cell cultures

Bacterial cultures were incubated at 30ºC to early exponential phase of growth (OD_{600} ~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 of NaCl $liter^{-1}$].

Finally, cells were resuspended in U buffer [10 mM sodium phosphate (pH7.0), 0.1 mM $MgSO_4$] to an OD_{600} of 2.5 – 3.0. Simazine was added at a final concentration of 0.06 mM to cell suspensions (6 ml), which were further incubated at 30ºC. Aliquots (0.8 ml) were taken at 15 minute intervals and centrifuged for 1 min at 13000 x g. The simazine concentration was determined by measuring the absorbance at 225 nm (Hernández *et al.*, 2008a). All growth experiments were done in triplicate.

Results

Isolation of simazine-degrading bacteria

Seventeen *Stenotrophomonas* strains, isolated by enrichment from two agricultural soils with histories of simazine application and from a control soil of central Chile, were able to grow on simazine as the sole nitrogen source. Seven strains were obtained from the avocado plantation soils, the same amount was obtained from the persimmon plantation soils and three strains were obtained from the control soil, *i.e.*, the soil that had no history of exposure to simazine.

Identification of bacterial isolates

The 16S rDNA genes of most of the isolates were sequenced and analysed. The sequences of the 16S rDNAs from these strains were aligned with reference sequences from the EMBL database. All the strains isolated were classified as *Proteobacteria*, class *Gammaproteobacteria*, order *Xanthomonadales*, family *Xanthomonadaceae* and most similar to species of *Stenotrophomonas*. 16S rRNA gene sequence analyses indicate that these seventeen strains possessed >99% similarity with the type strain of *S. maltophilia* (LMG 958^T, CCUG 5866-T) (Fig. 1). Conserved house-keeping genes, gyrase (topoisomerase type II) subunit B (*gyrB*) gene and the DNA-dependent RNA polymerase, sigma 70 (sigma D) factor (*rpoD*) gene sequences were analysed by PCR-amplification and comparative sequence analyses. Sequence similarities between the *gyrB* sequence of the *Stenotrophomonas* strains and those of *S. rhizophila* (e-p10, CCUG 54943-T) and *S. chelatiphaga* (LMP-5, CCUG 57178) were 93.8 and 89.8, respectively (Fig. 2a). Sequence similarities between the *rpoD* gene sequence of our *Stenotrophomonas* strains and those of *S. maltophilia* (LMG 958, CCUG 5866-T) and *S. chelatiphaga* (LMP-5, CCUG 57178)

were 90.6 and 89.6, respectively (Fig. 2b). The sequence similarities between the two house-keeping genes of the isolates and all published species of *Stenotrophomonas* were below 94% for *gyrB* and below 91% for *rpoD*.

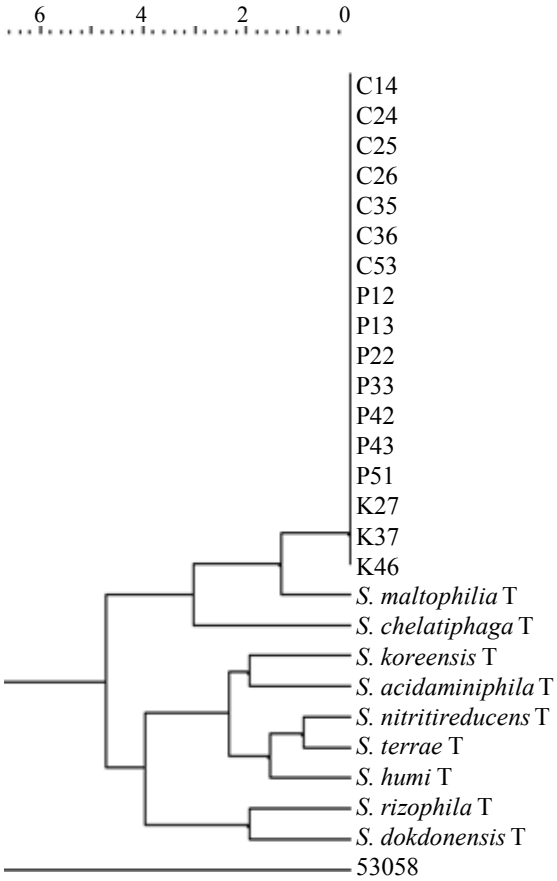

Figure 1. Dendrogram of estimated phylogenetic relationships between the strains isolated and related *Stenotrophomonas* species based on comparisons of partial 16S rRNA gene sequences. The dendrogram was generated using UPGMA cluster analysis. The scale represents percentage sequence differences.

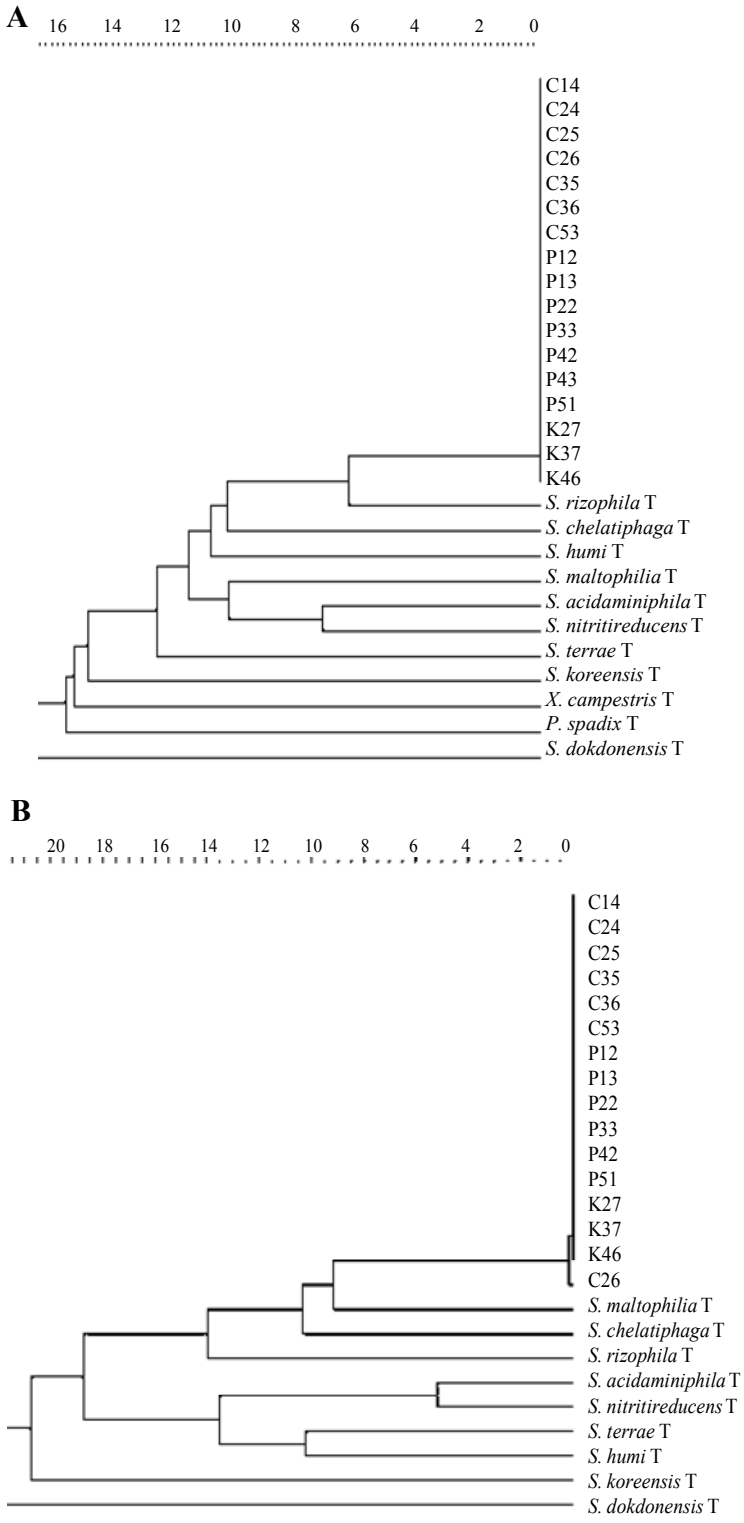

Figure 2. Dendrogram of estimated phylogenetic relationships between the two house-keeping genes of the isolates and all published species of *Stenotrophomonas*. A, Gyrase subunit B (*gyr*B). B, RNA polymerase, sigma 70 (sigma D) factor (*rpoD*). The dendrogram

was generated using UPGMA cluster analysis. The scale represents percentage sequence differences.

Analysis of *atzA, atzB, atzC, atzD, atzE* and *atzF* genes

The *atzA, atzB, atzC, atzD, atzE and atzF* genes that encode the enzymes of the *s*-triazine upper and lower catabolic pathways were detected by PCR-amplification. Among the seventeen strains of *Stenotrophomonas* sp. isolated, only strains P13, P33, P43 and C53 harboured the *atzA*, *atzB, atzC, atzD*, *atzE* and *atzF* genes. All other isolates possessed, at least, one of the *atz* genes of the catabolic pathway (Table 2).

Table 2. Presence of *atz*A, *atz*B, *atz*C, *atz*D, *atz*E and *atz*F genes of simazine degrading bacteria isolated.

Soil	Strain	CCUG	*atz* genes
Persimmon	C14	CCUG 50358	nd
Persimmon	C24	CCUG 50360	*atz*B
Persimmon	C25	CCUG 50361	*atz*B, *atz*E, *atz*F
Persimmon	C26	CCUG 50362	*atz*B, *atz*D, *atz*E, *atz*F
Persimmon	C35	CCUG 50364	nd
Persimmon	C36	CCUG 50365	*atz*B, *atz*E, *atz*F
Persimmon	C53	CCUG 50368	*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F
Avocado	P12	CCUG 50371	nd
Avocado	P13	CCUG 50372	*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F
Avocado	P22	CCUG 50374	*atz*B, *atz*C, *atz*E, *atz*F
Avocado	P33	CCUG 50377	*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F
Avocado	P42	CCUG 50379	*atz*D
Avocado	P43	CCUG 50380	*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F
Avocado	P51	CCUG 50381	*atz*A, *atz*B, *atz*D, *atz*F
Control	K27	CCUG 50383	*atz*A, *atz*C, *atz*D, *atz*F
Control	K37	CCUG 50384	*atz*C, *atz*D, *atz*E
Control	K46	CCUG 53066	*atz*B, *atz*F

nd. not detected

Characterization and identification of simazine-degrading bacteria.

The degradation of simazine by two *Stenotrophomonas* sp. strains that possess all the *atz* genes was analyzed. Cells grown on simazine were harvested, washed and resuspended in buffer with simazine (0.06 mM) as only nitrogen source. *Stenotrophomonas*

maltophilia strains P13 and P33 were able to degrade > 50% simazine after one-hour (Fig. 3).

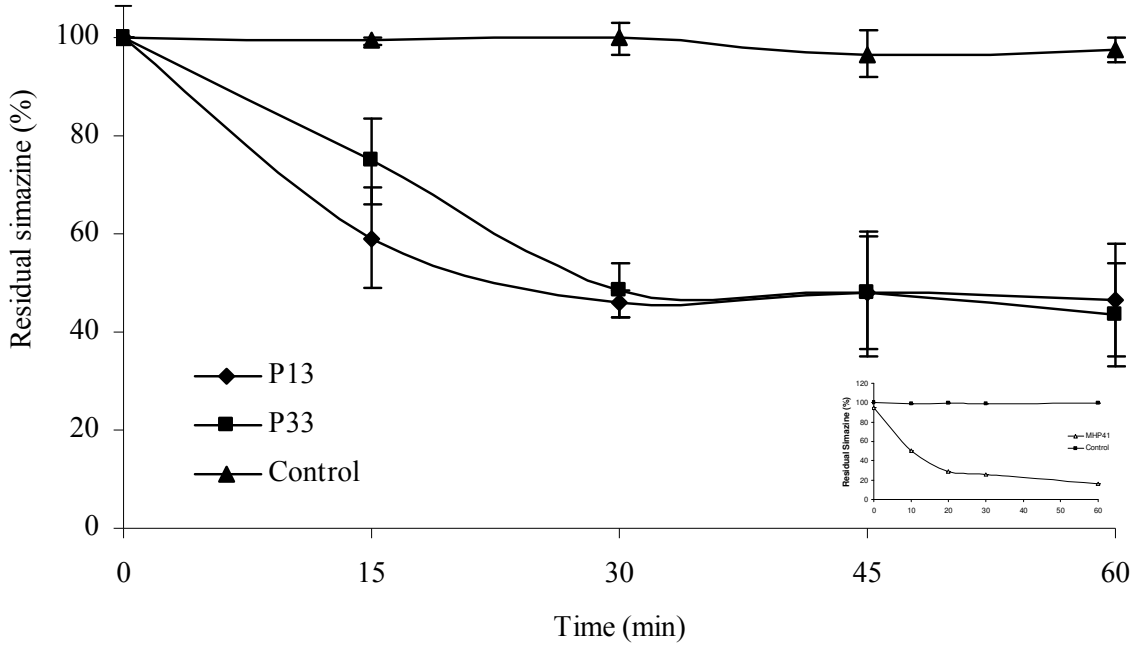

Figure 3. Simazine degradation in two strains of *Stenotrohpomonas* sp. The strains were grown in minimal medium with simazine at saturation and resuspended in U buffer (OD_{600}: 0.25). The control was U buffer without cells. The samples were incubated in simazine (0,06 mM) and analysed by spectrophotometry. Insert figure shows a similar experiment with the strain *Pseudomonas nitroreducens* MHP41 (Hernández *et al.*, 2008a).

Discussion

The aim of the present study was to characterize strains of *Stenotrophomonas* spp. involved in simazine degradation. *s*-Triazine degrading bacterial strains have been isolated from herbicide polluted soils (Mandelbaum *et al.*, 1995; Ralebitso *et al.*, 2002) and ground water (Tappe *et al.*, 2002). Mixed communities of microorganisms (De Souza *et al.*, 1998a) and strains, such as *Pseudomonas* sp. (Mandelbaum *et al.*, 1995), *Pseudoaminobacter* sp. (Topp *et al.*, 2000b), *Agrobacterium* sp. (Struthers *et al.*, 1998), *Nocardioides* sp. (Topp *et al.*, 2000a) and *Stenotrophomonas maltophilia* (Rousseaux *et al.*, 2001; Marecik *et al.*,

2008) with atrazine-degrading capabilities have been isolated from soils treated with atrazine.

In the present work, a genetic characterization by 16S rRNA gene sequence analyses as well as DNA gyrase subunit B (*gyrB*) and RNA polymerase subunit D (*rpoD*) "housekeeping" genes, allowed the differentiation and identification for seventeen isolates identified as *Stenotrohpomonas*. All the isolates presented in this study are able to growth using simazine as the only nitrogen source. Also, the present study evaluated the simazine degradation by the *Stenotrophomonas maltophilia* strains P13 and P33. There are few studies who present the potential of *Stenotrophomonas maltophilia* in the atrazine degradation and in the *atz* gene detection. Rousseaux et al. (2001) presented a *Stenotrophomonas maltophilia* strain Lous 3-4 isolated in France, this strain is able to dechlorinated the atrazine and posseses just the *atzA* gene. To the other hand, a *Stenotrophomonas maltophilia* strain BM4 isolated from the rhizosphere of sweet flag is able to degrade 83.5% of atrazine after 15 days of culture (Marecik *et al.*, 2008). This is the first report of *Stenotrophomonas maltophilia* strains able to degrade simazine and that present the *atzA, atzB, atzC, aztD, atzE* and *atzF* necessary for thecomplete mineralization of the herbicide.

Due to the results obtained in the present work according to the 16S rRNA gene sequence as well as the *gyrB* and *rpoD* analysis for the identification of the *Stenotrophomonas* sp. samples suggest that our isolated strains probably comprise a different species. Analysis of house-keeping genes *gyrB* and *rpoD* exhibited an effective species-level differentiation for *Stenotrophomonas*, which can be applied, in combination with 16S rRNA gene sequence analysis to provide an MLSA strategy for genotypic analysis for reliable identifications of isolates from clinical and environmental samples. Multi locus sequence analysis (MLSA) is increasingly seen as offering an alternative, more flexible way of comparing bacteria, towards the development of a species concept.

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Chapter 4

Part I

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

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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 μ=0.10 h^{-1}, reaching a high biomass of 4.2 x 10^8 colony forming units (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 Biolog microplate system and comparative sequence analyses of the 16S rRNA gene. Amplified ribosomal DNA restriction analysis (ARDRA) allowed the differentiate strain MHP41 from *Pseudomonas* sp. ADP. The comparative 16S rRNA gene sequence analyses suggested that strain MHP41 is closely related with *P. nitroreducens* and *P. 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 post-emergence 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 $\mu g\ L^{-1}$ and 3.0 $\mu g\ L^{-1}$ 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 tons 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) were added to 90 mL of AM medium (AM) (Rousseaux *et al.*, 2001) with added simazine as the sole nitrogen source. The medium contained (per L): 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7\ H_2O$; 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 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} $CuSO_4 \cdot 5H_2O$; 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 $FeSO_4 \cdot 7\ H_2O$ (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 (Ausubel *et al.*, 1999). Enrichments were incubated at 28ºC with agitation (150 r.p.m.). Enrichment cultures were sub-cultured 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 ºC. 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 °C and 42 °C. AM agar with simazine (3.0 mM) and Tryptic Soy (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 30ºC as described by García-Gonzalez *et al.* (García-González *et al.*, 2003), to the exponential phase ($turbidity_{600}$ ~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 of NaCl L^{-1}]. Finally, cells were resuspended to a $turbidity_{600}$ of 2.5 - 3.0 using 10 mM sodium phosphate (pH7.0), 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 ºC. Aliquots of cell suspensions were taken after different incubation times and centrifuged for 1 min at 16,000 x g. The simazine concentration was determined by measuring the absorbance at 225 nm. Degradation assays were performed in triplicate.

Analyses of *atz* genes

The *atzA, atzB, atzC, atzD, atzE and atzF* genes were assessed by 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.

ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA) was performed on the bacterial strains MHP41 and *Pseudomonas* sp. ADP (Mandelbaum *et al.*, 1995). A bacterial colony was suspended in 100 µL sterile deionized H_2O and heated at 94ºC 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), using the protocols of the manufacturer. ARDRA profiles (2-5 µL of digested 16S rRNA gene products) were separated by electrophoresis (MiniProtean, Biorad) in 8% polyacrylamide gel in Tris borate EDTA buffer, at 130V, for 60 minutes, 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'-CCCCTTTCCCTCACGGTAC-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, with the protocols of the manufacturer (Applied Biosystems, Inc., Foster City, CA.). Sequence data were edited manually and compared to 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, which 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 approximately 30ºC. Strain MHP41 was able to grow using simazine or atrazine as the only source of nitrogen. 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 carbon source and simazine as the sole nitrogen source, at 30ºC, reached 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 one hour (Fig. 1).

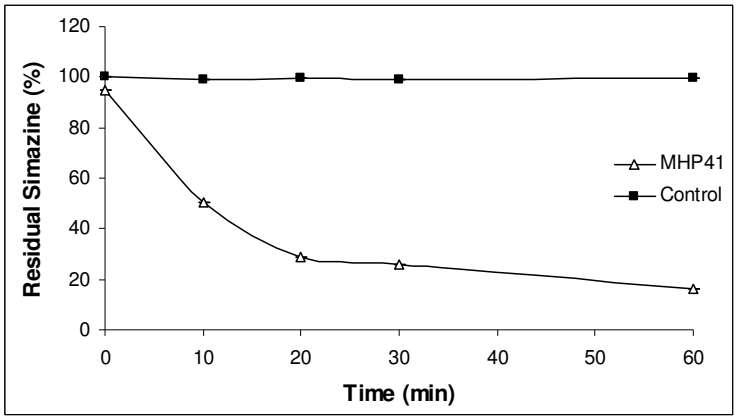

Figure 1. Simazine degradation by *Pseudomonas* sp. MHP41. Resting cells of a culture previously grown in AM broth with simazine were incubated with simazine (0.06 mM). The control was phosphate buffer supplemented with $MgSO_4$. Each value is an average of three independent experiments.

Detection of *atz* catabolic genes

The *atzA, atzB* and *atzC* genes, encoding the "upper" *s*-triazine catabolic pathway, were detected by PCR-amplification using specific set of primers (De Souza *et al.*, 1998b). The expected sizes of PCR products were observed: *atz*A: 0.5-kb; *atz*B: 0.5-kb; *atz*C: 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: *atz*D: 0.5-kb; *atz*E: 0.2-kb; and *atz*F: 0.3-kb *atzF* are shown in Fig. 2.

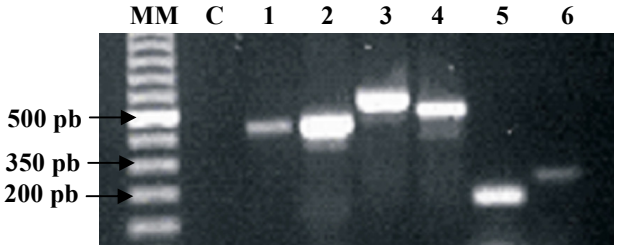

Figure 2. Detection by PCR of *atzA, atzB, atzC, atzD, atzE* and *atzF* genes for simazine degradation in *Pseudomonas* sp. MHP41. PCR-amplification products: *atzA* (lane 1)*, atzB* (lane 2)*; atzC* (lane 3)*; atzD* (lane 4)*; atzE* (lane 5)*; atzF* (lane 6). X molecular mass (MM) markers (Roche, Basel).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

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 maltose. The isolate MHP41 was tentatively identified as a *Pseudomonas* sp. by the Biolog identification system, with a similarity index of 0.76 with *P. nitroreducens* and *P. azelaica* (>0.50 is considered an acceptable match) (Table 1). ARDRA electrophoretic profiling (using restriction endonucleases *Msp1* or *Hha1*) 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).

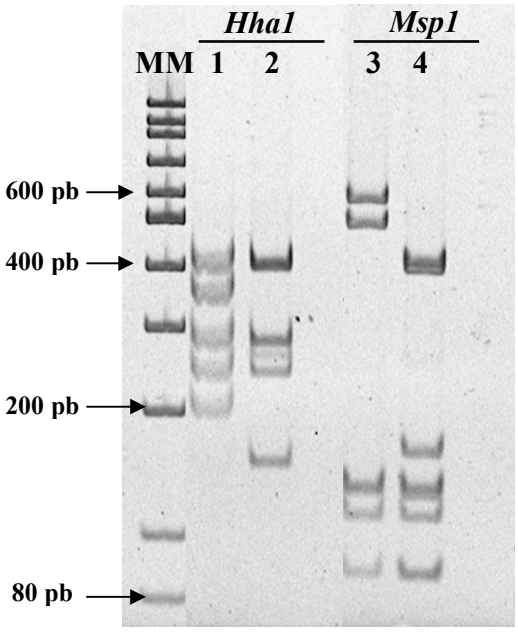

Figure 3. ARDRA patterns of *Pseudomonas* sp. MHP41 and *Pseudomonas* sp. ADP. 16S rRNA gene PCR-amplification products of *Pseudomonas* sp. ADP (lines 1 and 3) and *Pseudomonas* sp. MHP41 (lines 2 and 4) were digested with *HhaI* and *MspI*. 100 pb molecular mass (MM) markers, (Fermentas, Ontario) are shown at the left side.

Comparative 16S rRNA gene sequence analysis identified strain MHP41 as a genus of *Pseudomonas*, with a sequence most similar to *P. 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.

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

C Sources			
Carboxylic acids	**Amino acids**	**Polymers**	**Amides and Amines**
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	**Carbohydrates**	
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	**Esters**	
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 *P. nitroreducens* MHP41 16S rRNA gene (EBI Accession number: AM922106) with the respective sequences from other *Pseudomonas* species

		P. nitroreducens MHP41 (CCUG 50378) 16S rRNA gene	
P. multiresinovorans	(IpA-1 = CCUG 51655-T)	X96787	99.8
P. nitroreducens	(DSM 14399 = CCUG 12538-T)	AM088474	99.7
Pseudomonas sp.	(ADP = CCUG 53068)	AM088478	98.9
P. citronellosis	(DSM 50332 = CCUG 17933-T)	Z76659	98.4
P. alcaligenes	(LMG 1224 = CCUG 1425-T)	Z76651	97.3
P. aeruginosa	(ATCC 27853 = CCUG 17619)	AY268175	96.9
P. pseudoalcaligenes	(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 *E. coli* 16S rRNA gene sequence positions 28 – 1490.

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 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 with the species *P. nitroreducens* and *P. multiresinovorans* and distinct from *P. citronellol*is 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 to *P. nitroreducens*.

The recognition that soil organisms can influence ecosystem processes has lead 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 a *s*-triazine-degrading bacterial strain isolated in South America. This study further establishes the world wide spread 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; Seeger *et al.*, 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 for degrading a variety of different contaminants (Díaz, 2004). The genus *Pseudomonas* is present in ecologically diverse niches, playing important roles in the bio-geochemical 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 and Parsek, 2003; Wackett, 2003). Strains of *Pseudomonas* able to degrade atrazine and to use it as the sole source of nitrogen have been isolated previously (Cook & Hütter, 1984; Mandelbaum *et al.*, 1995).

Interestingly, this study shows that the novel *Pseudomonas* sp. MHP41 reaches high biomass using simazine as sole nitrogen source ($turbidity_{600}$ 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 reach a $turbidity_{600} < 0.6$, whereas *Arthrobacter* strain AD1 attains a $turbidity_{600} < 0.9$ (Radosevich *et al.*, 1995;

Strong *et al.*, 2002; Cai *et al.*, 2003; Iwasaki *et al.*, 2007). Although *Pseudomonas* sp. MHP41 showed with simazine as sole N source a relatively high growth rate ($\mu = 0.10$) h^{-1}, *Pseudomonas* sp. ADP using atrazine as N source exhibited faster growth ($\mu = 0.22$ 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 posses 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 *Pseudaminobacter* 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-Ic posses 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*) have been detected (Cheng *et al.*, 2005). As diverse *s*-triazine degrading microbes have several catabolic *atz* gene combinations, the *atz* genes seem 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 source of nitrogen and yielding high biomass. *Pseudomonas* sp. MHP41 posseses 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.

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Chapter 4

Part II

Genetic and systematic characterization of an efficient simazine-degrading bacterium, *Pseudomonas nitroreducens* MHP41

Abstract

Strain MHP41 is a *s*-triazine-degrading bacterium, isolated from agricultural soil in Chile, which has been used for soil remediation. Strain MHP41 is able to grow, rapidly degrading and using simazine as sole nitrogen source. Using a resting cell assay and HPLC quantification of the herbicide, fast degradation of more than 90% of simazine by MHP41 cells was observed. This bacterium was allocated in the genus *Pseudomonas* by phenotypic (Biolog) and 16S rRNA gene sequence analyses in a previous study. The aims of this study were the genetic characterization and detailed systematic classification of strain MHP41. Physiological and biochemical characterizations of strain MHP41, using API 20NE and API ZYM and other phenotyping assays gave best matches with *P. nitroreducens*. The rod-shaped morphology was observed by transmission electronic microscopy. Multi-locus sequence analyses (MLSA), utilizing *gyrB* and *rpoB* "housekeeping" genes, as well as 16S-23S rRNA inter-genic spacer (IGS-1) regions, further differentiated and identified strain MHP41 as a strain of *P. nitroreducens* . Partial sequences of the sequences of the six catabolic genes,*atzA*, *atzB*, *atzC*, *atzD*, *atzD* and *atzF*, of strain MHP41 were observed to be identical or highly similar to the respective *atz* sequences of *Pseudomonas* sp. ADP and other *s*-triazine-degrading strains. The *atzD*, *atzE* and *atzF* were transferred by conjugation from strain MHP41 to *P. putida* KT2442, indicating these genes to be mobile; probably located on a plasmid. MHP41 (CCUG 50378) is the first *P. nitroreducens* strain reported to degrade *s*-triazine compounds.

Introduction

Herbicides such as *s*-triazines are used extensively for the control of weeds in agriculture. The USA is the main herbicide consumer worldwide, with an annual application of more than 200,000 tons. The annual use of herbicides in South America was approximately 60,000 tons between 1990 and 2000 (Hernández *et al.*, 2008a). The mobility of *s*-triazine has been a cause of increasing concern in several countries due to the potential contamination of surface and groundwater (Flores *et al.*, 2009).

Microorganisms play important roles in biodegradation, including the degradation of herbicides and their removal from the environment (Mandelbaum *et al.*, 1995; Rousseaux *et al.*, 2001; Hernández *et al.*, 2008a; Hernández *et al.*, 2008b). Native *s*-

triazine-degrading bacterial strains have been isolated in soil environments from diverse regions (Mandelbaum *et al.*, 1995; Rousseaux *et al.*, 2001; Hernández *et al.*, 2008b). *Pseudomonas* sp. strain ADP is the best characterized atrazine-degrading strain and one of few strains of *Pseudomonas* that have been reported (Mandelbaum *et al*., 1995; Martinez *et al*., 2001). Recently, an interesting *s*-triazine-degrading strain of *Pseudomonas* sp. was isolated from agricultural soil in central Chile (Hernández *et al.*, 2008b). The *s*-triazine degradation pathway has been characterized (Fig. 1).

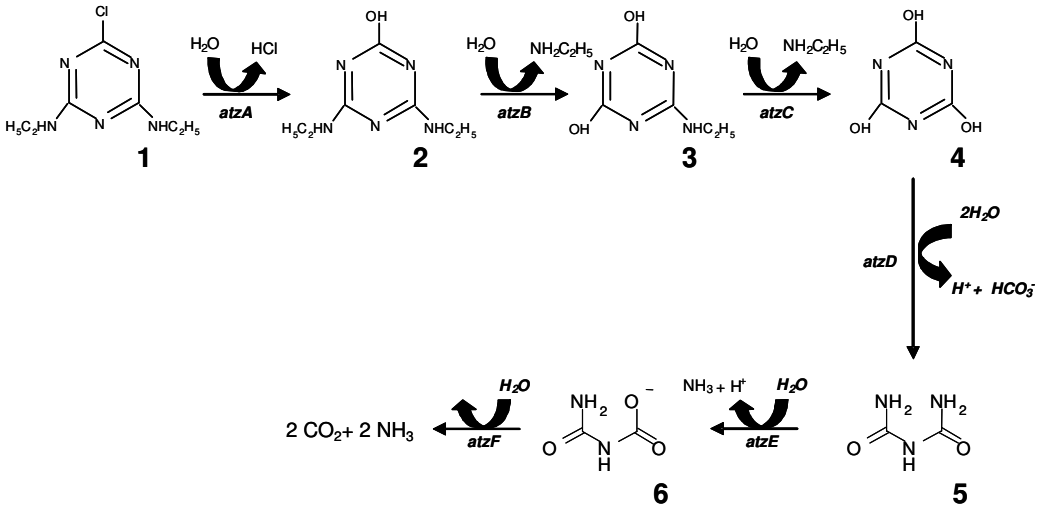

Figure. 1. The *s*-triazine catabolic pathways of strain MHP41. Upper pathway substrate and metabolites: simazine (1), hidroxysimazine (2), N-etilammelide (3) and cyanuric acid (4). Lower pathway substrate and metabolites: cyanuric acid (4), biuret (5), allophanate (6). Enzymes encoded by *atz* genes: atrazine chlorohydrolase (AtzA); hydroxyatrazine ethylaminohydrolase (AtzB); N-isopropylammelide isopropylaminohydrolase (AtzC); cyanuric acid amidohydrolase (AtzD); biuret hydrolase (AtzE); allophanate hydrolase (AtzF).

For the typing and identification of bacteria, a variety of genotypic-based methodologies have been described. Sequencing of the 16S ribosomal RNA (16S rRNA) gene has served as an important tool for estimating phylogenetic relationships between bacteria and the database of 16S rRNA gene sequences comprises the largest collection of a phylogenetic marker (Yarza *et al.*, 2008). The features of this molecular target are useful as a phylogenetic tool and for identifying new environmental bacterial strains (von

Wintzingerode *et al.*, 1997; Clarridge, 2004; Janda and Abbott, 2007). However, the conserved nature and limited resolution of the 16S rRNA gene sequence does not allow the differentiation of closely related species (Clarridge, 2004). Therefore, identifications of strains and isolates from a variety of taxa have been assessed by comparative nucleotide sequence analyses of less-conserved "housekeeping" genes of the core bacterial genome (Konstantinidis and Tiedje, 2005), such as the genes for DNA gyrase β-subunit (*gyrB*) (Yamamoto and Harayama, 1998) and RNA polymerase β-subunit (*rpo*B) (Tayeb *et al.*, 2005), as well as the 16S-23S rRNA inter-genic spacer (IGS-1) region (Guasp *et al.*, 2000).

The aims of this study were to characterize and classify the simazine-degrading strain MHP41 isolated from agricultural soil in central Chile (Hernández *et al.*, 2008b). *S*train MHP41 represents a useful model system for studying the degradation of *s*-triazine herbicides and has been applied for remediating *s*-triazine-contaminated soils (Morgante et al., 2010).

Results and Discussion

MHP41 growth and degradation of simazine

Strain MHP41 was isolated from an agricultural soil of central Chile, using selective enrichment, with simazine added to cultivation medium as a sole nitrogen source (Hernández *et al.*, 2008b). The growth of strain MHP41 in minimal medium with simazine as the sole nitrogen source and glucose as carbon source is depicted in Figure 2. Strain MHP41 showed fast growth with high biomass at stationary phase, which was in agreement with observations of MHP41 growth with simazine and citrate (Hernández *et al.*, 2008b).

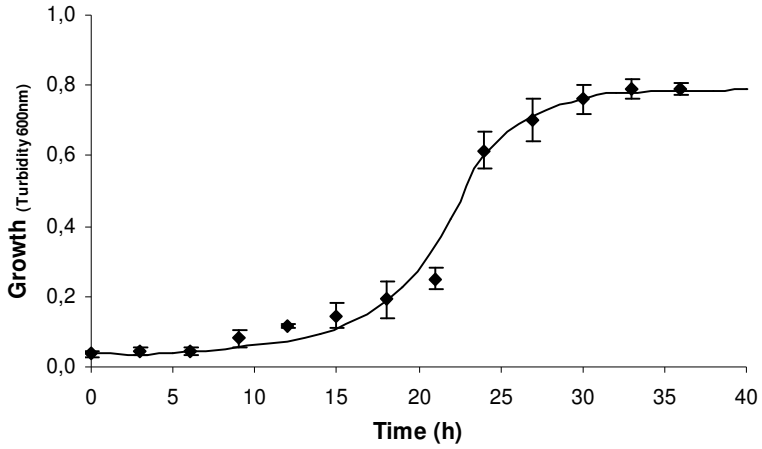

Figure 2. Growth of *Pseudomonas* nitroreducens MHP41 in AM broth with glucose as sole carbon source and simazine (0.5 mM) as the sole nitrogen source. The values are the mean of three independent experiments. Bars represent standard deviations of the averages.

Simazine degradation by strain MHP41 was analyzed. Resting cells were incubated with simazine (0.5 mM), and the herbicide was quantified by HPLC. Figure 3 shows the rates of simazine degradation by strain MHP41. The efficient degradation rate measured by HPLC (Fig. 4) correlates with degradation reported previously (Hernández *et al.*, 2008b).

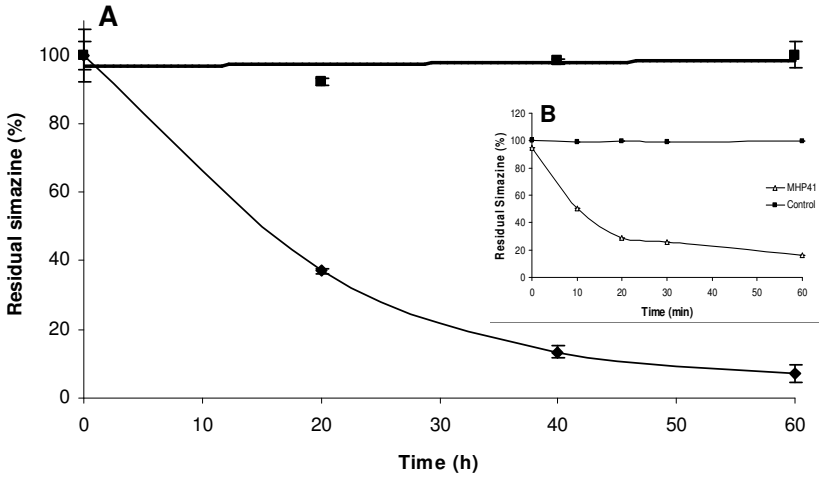

Figure 3. Simazine degradation by strain MHP41. The cells grown in minimal medium with simazine were incubated in phosphate buffer with simazine (0.06 mM). The control was phosphate buffer without cells. Bars represent standard deviations of the averages of three independent experiments. A. Degradation of simazine determined by HPLC. B. Insert figure shows a similar experiment in that simazine degradation was measured by spectrophotometer (Hernández *et al.*, 2008b).

Taxonomic characterization and identification

Strain MHP41 is a Gram-negative bacterium with rod-shaped morphology, as observed by transmission electron microscopy (Fig. 4). A detailed phenotypic characterization of strain MHP41 was carried out, using API 20NE, API ZYM, as well as customized tests (Table S1). The complete phenotypic data profile for strain MHP41 may be seen at: http://www.ccug.se. Probabilistic identifications based upon the phenotypic

profile gave excellent matches with *P. nitroreducens* (CCUG 12538-T) and *P. multiresinovorans* (CCUG 51655-T). Table S1 shows the phenotypic variation between MHP41 and reference strains of *P. multiresinovorans, P. nitroreducens, P. citronellolis, Pseudomonas* sp. ADP and *P. aeruginosa.*

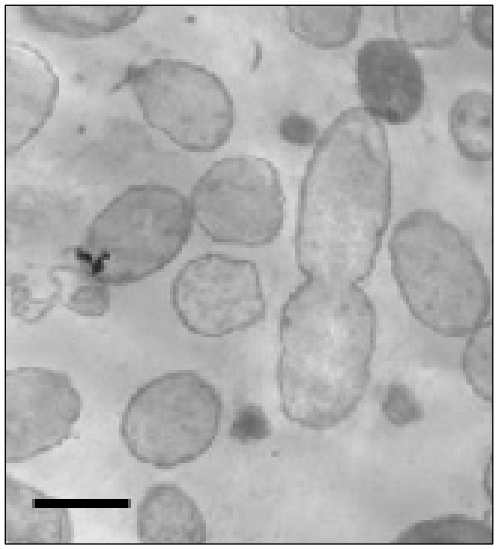

Figure. 4. Cell morphology of simazine-degrading *Pseudomonas nitroreducens* MHP41 observed by transmission electronic microscopy. Ultra-thin sections of the bacterial cells grown using glucose as sole carbon source and simazine as sole nitrogen source. Bar represents 1 μm.

The *s*-triazine-degrading strain MHP41 was initially allocated to the genus *Pseudomonas*, by phenotypic (Biolog) and 16S rRNA gene sequence analyses (Hernández *et al.*, 2008b). Importantly, ARDRA and comparative 16S rRNA gene sequence analyses confirmed strain MHP41 to be distinct from *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995), the best-characterised *s*-triazine-degrading strain of *Pseudomonas* (Hernández *et al.*, 2008b). Strain MHP41 and *Pseudomonas* sp. ADP were observed to possess 16S rRNA gene sequences with a similarity of 98.9%; Stackebrandt and Ebers (2006) have suggested that strains with 16S rRNA gene sequence similarities less than 99.0-98.7% are most likely different species. Many species of *Pseudomonas* exhibit 16S rRNA gene sequence similarities greater than 98.9% (Moore *et al.*, 1996; Anzai *et al.*, 1997; Anzai *et al.*, 2000).

The sequence of the PCR-amplification product was determined (2,411 nucleotide positions), including the 16S rRNA gene (1509 nucleotide positions, excluding the 5'-terminus complementary to the forward PCR-primer), the IGS-1 region between the 16S and 23S rRNA genes (473 nucleotide positions) and the 5'-region (429 nucleotides) of the 23S rRNA gene. Comparative sequence analysis with 16S rRNA gene sequences of reference strains of *Pseudomonas* species revealed similarities of 99.8, 99.7, and 98.4%, between the sequence of strain MHP41 and those of the type strains of *P. multiresinovorans*, *P. nitroreducens* and *P. citronellolis*, respectively (Table 1). Comparisons of the 16S rRNA gene sequence of strain MHP41 with the sequences of the type strains of all other species of *Pseudomonas* exhibited sequence similarities below 98.0%. Such levels of 16S rRNA gene sequence similarity reflected the closest phylogenetic relationships between strain MHP41 and *P. multiresinovorans* and *P. nitroreducens* (Table 1). During the course of this study, Lang et al. (2007) demonstrated unequivocally, through an extensive polyphasic analysis, that the type strains of *P. multiresinovorans* and *P. nitroreducens* are strains of the same species and that *P. multiresinovorans* should be regarded as a later heterotypic synonym of *P. nitroreducens*. Thus, on the basis of these data, strain MHP41 also was concluded to be a strain of *P. nitroreducens*.

In this study, based on phenotypic and genotypic higher-resolution analyses, strain MHP41 was classified as a strain of *P. nitroreducens* (Table S1). The IGS-1 sequence of strain MHP41 was determined and compared with the IGS-1 region sequences of the type strains of the most closely related *Pseudomonas* species, *P. nitroreducens* strain CCUG 12538^{T} and *P. multiresinovorans* CCUG 51655^{T} (Table 1). IGS-1 comparative sequence analyses provide a measure of much higher resolution between closely related species than the more conserved 16S rRNA gene sequences for determining the relationships between closely related species. The IGS-1 of strain MHP41, as well as *P. nitroreducens* and *P. multiresinovorans* exhibited the typical sequence organization between the 16S rRNA and 23S rRNA genes, in the sequence format: 16S rRNA - IGS-V1 - tRNA^{Ile} - IGS-V2 - tRNA^{Ala} - IGS-V3 - 23S rRNA. The IGS-1 sequence similarities between strain MHP41 and *P. nitroreducens* (475 nucleotides) and *P. multiresinovorans* (475 nucleotides) were lower than the similarities observed for 16S rRNA gene sequence comparisons (99.2 and

98.9%, respectively) (Table 1). Omitting the conserved tRNA gene sequences from the comparisons did not change the similarities for the IGS-1 region sequences between strain MHP41, *P. multiresinovorans* and *P. nitroreducens*, although the sequence similarities to the IGS-1 of other species of *Pseudomonas* were reduced by 1.6-3.8% (data not shown). The IGS-1 sequence similarity between the type strains of *P. nitroreducens* and *P. multiresinovorans* was observed to be 99.6%. Such an IGS-1 sequence similarity supports the conclusions of Lang *et al*. (2007) that *P. nitroreducens* and *P. multiresinovorans* are the same species. Although the level of IGS-1 sequence similarity necessary to definitively delineate one species from another has not been conclusively defined, the values of sequence similarities observed in this study between strain MHP41 and *P. nitroreducens* (type strain), *P. multiresinovorans* and other species of *Pseudomonas* conform with the observations of Guasp et al. (2000) for *P. stutzeri* genomovars, or genotypic "species".

Conserved house-keeping genes, gyrase (topoisomerase type II) subunit B (*gyrB*) gene and the DNA-dependent RNA polymerase *β*-subunit (*rpoB*) gene sequences were analysed by PCR-amplification and comparative sequence analyses. Sequence similarities between the *gyrB* sequence of strain MHP41 and those of *P. multiresinovorans* and *P. nitroreducens* were 94.8 and 94.6, respectively. Sequence similarities between the *rpoB* gene sequence of strain MHP41 and those of *P. multiresinovorans* and *P. nitroreducens* were 96.5 and 97.0, respectively. The sequence similarities between the two house-keeping genes of strain MHP41 and all other species of *Pseudomonas* were below 90% for *gyrB* and below 94% for *rpoB* (Table 1).

Table 1. Multi-locus sequence similarities of 16S rRNA, IGS-1, *gyr*B and *rpo*B of *P. nitroreducens* MHP41 with the respective gene sequences from other *Pseudomonas* species.

Pseudomonas species	Strain	*P. nitroreducens* MHP41 (CCUG 50378)							
		16S rRNA		IGS-1		*gyrB*		*rpoB*	
P. nitroreducens	DSM 14399 = CCUG 12538-T	99.7	AM088474	99.2	AM922483	94.6	FN568272	97.0	FN568267
P. multiresinovorans	IpA-1 = CCUG 51655-T	99.8	X96787	98.9	AM922484	94.8	FN568273	96.5	FN568268
P. citronellosis	DSM 50332 = CCUG 17933-T	98.4	Z76659	nd	-	87.1	AB039452	93.8	FN568270
Pseudomonas sp.	ADP = CCUG 53068	98.9	AM088478	nd	-	90.8	FN568274	93.5	FN568269
P. aeruginosa	ATCC 27853	96.9	AY268175	96.9	L28150	88.7	AB039386	91.8	AJ717442
P. alcaligenes	LMG 1224	97.3	Z76651	nd	-	87.8	AB039388	91.2	AJ279966
P. pseudoalcaligenes	ATCC 17440	96.7	Z76666	79.9	AJ279245	83.9	AB039397	90.3	AJ717430
P. mendocina	ATCC 25411	95.4	L28159	84.0	L28159	87.7	AJ633103	91.9	AJ279967
P. stutzeri	ATCC 17588	96.4	U26262	96.2	AJ251910	84.9	AB039393	87.6	AJ279962
P. putida	KT2440 = DSM 6125	95.5	AE015451	93.0	AE015451	82.2	AE015451	90.8	AE015451
P. fluorescens	Pf-5 = ATCC BAA-477	94.0	NC_007492	78-0	NC_007492	82.8	NC_007492	87.2	NC_007492

MHP41 IGS-1 (EBI Accession number: AM922106); *gyr*B, gyrase subunit B (EBI Accession number: FN568271); *rpo*B, RNA polymerase β-subunit (EBI Accessison number: FN568266)

Table S1. Phenotypic features differentiating *Pseudomonas* sp. MHP41 and related *Pseudomonas* species.

Test Panel	Phenotypic test	*Pseudomonas* sp. MHP41 CCUG 50378	*Pseudomonas* sp. ADP CCUG 53068	*P. multiresinovorans* CCUG 51655-T	*P. nitroreducens* CCUG 12538-T	*P. citronellosis* CCUG 17933-T	*P. aeruginosa* CCUG 551-T
OX	Hemolysis, horse	1	1	1	1	1	3
	Odour	1	1	1	1	1	3
	NA 42 C-growth	1	4	3	3	4	5
	Motility HD/30 C	3	1	5	4	4	5
	Motility OF	4	1	4	3	4	5
OF	Maltose	1	1	2	1	1	3
	D-Fructose	3	1	4	3	1	1
	D-Xylose	1	5	1	1	3	5
	Fluorescin	1	5	1	1	5	5
	Tween 80	1	1	1	1	1	3
	NaCl 6% – growth	1	2	1	1	2	5
DEC	Urease, Christ	1	1	1	1	1	3
	Gelatine Kohn	1	1	1	1	1	5
ESC	Acetamide	1	1	1	1	1	4
	NaCl 4.5% – growth	4	2	2	3	3	5
ASSIM	Adipate	5	5	5	5	1	4
	Phenylacetate	5	5	5	3	5	1
API 20 NE	D-Mannitol	1	1	1	1	1	5
	N-Ac-Glucosamine	3	1	1	1	1	2
	Adipate	5	5	5	5	1	5
	Phenylactate	5	5	5	4	5	1
API ZYM	Phosph Allcaline	4	4	4	5	5	2
	Esterase (C-4)	3	3	3	2	3	4
	Lipase (C-14)	1	1	1	1	1	3
	Trypsin	3	4	2	4	4	1
	Phosphoamidase	1	5	3	4	2	2

Test Panel and Phenotypic Tests are defined in the CCUG Identification worksheets, described at: http://www.ccug.se/default.cfm?navID=160

Phenotypic Tests data coding: 1 = negative; 2 = probably negative; 3 = weakly positive; 4 = postitive; 5 = strongly positive.

Catabolic gene characterization

The six catabolic *atz* genes of strain MHP41 (Hernández *et al.*, 2008b) were further characterized by sequence determinations. Sequence analyses of *atzA, atzB, atzC, atzD, atzE and atzF* revealed that these *s*-triazine degrading gene sequences in strain MHP41 are highly similar to the respective genes of other *s*-triazine-degrading bacteria isolated in different regions (Table 2). The analyses showed high similarities (100% in most of the cases) with genes of already well-characterized *s*-triazine-degrading bacteria (Table 2). The *atz* genes of the upper *s*-triazine pathway from strain MHP41 were identical (100%) to those of the model bacterium *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995; Martínez *et al.*, 2001), *Arthrobacter* sp. strain AD1 (Cai *et al.*, 2003), *Arthrobacter aurescens* strain TC1 (Strong *et al.*, 2002; Sajjaphan *et al.*, 2004) and *Chelatobacter heintzii* strain Cit1 (Rousseaux *et al.*, 2001) and other bacteria. Likewise, *atzD*, *atzE* and *atzF* of the lower *s*-triazine pathway of strain MHP41 were identical or highly similar to the genes of *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995; Martínez *et al.*, 2001) and *Athrobacter* sp. strain MCMB-436 (Vaishampayan *et al.*, 2007) (Table 2). The extent of the genes in the bacteria noted, as well as the range of different taxa exhibiting these genes suggests that these catabolic genes are mobile and easily transferred. Indeed, studies have shown that the catabolic genes atzA, atzB and atzC are conserved and widespread throughout the world (De Souza *et al.,* 1998; Rousseaux *et al*., 2001). Devers et al. (2007) isolated seven new atrazine-degrading bacteria, from an agricultural soil, comprising species of 5 different genera. Notably, no strains of *Pseudomonas* sp. were obtained. In point of fact, most strains that have been isolated and characterized, containing *atz* genes have been of the genera of *Arthrobacter* spp., *Nocardioides* spp. and other Gram-positive bacteria. Although Gram-negative, *Agrobacterium* spp., *Ralstonia* spp., *Herbaspirillum* spp., etc., have been isolated with *atz* genes, only three strains of *Pseudomonas* spp., have been reported, *i.e*., *Pseudomonas* sp. strain ADP, *Pseudomonas* sp. NRRL-B-12227 and *P. nitroreducens* strain MHP41.

Table 2. Comparative sequence analysis of *s*-triazine degradation genes *atzA, atzB, atzC, atzD, atzE* and *atzF* of *P. nitroreducens* MHP41 with *atz* genes sequences of other *s*-triazine degrading bacteria.

Bacteria	Strain	Plasmid	Origin	*Pseudomonas nitroreducens* MHP41 (CCUG 50378) Similarity (%) and accession number						References
				atzA	*atzB*	*atzC*	*atzD*	*atzE*	*atzF*	
				507*	500*	601*	526*	249*	306*	
Pseudomonas sp.	ADP	pADP-1	Agricultural soils, USA	100.0 U66917	100.0 U66917	100.0 U66917	110. U66917	100.0 U66917	99.7 U66917	(de Souza *et al.*, 1995; Mandelbaum *et al.*, 1995; Martínez *et al.*, 2001)
Arthrobacter sp.	AD1	**	Wastewater, China	100.0 AF543694						(Cai *et al.*, 2003)
Herbaspirillum sp.	B601	pHB1	Agricultural soils, Belarus	99.8 DQ089655	99.8 AY965854	100.0 AY965855				Unpublished
Beta proteobacterium	CDB21	nd	Agricultural soils, Japan	99.6 AB194097	100.0 AB194098					(Iwasaki *et al.*, 2007)
Arthrobacter nicotinovoran	HIM	pCR4	Sandy dune soil, New Zealand	99.6 AY650035	99.5 AY650036	99.5 AY650037				(Aislabie *et al.*, 2005)
Chelatobacter heintzii	Cit1	nd	Agricultural soils, France	99.6 AF364900	100.0 AF364901	100.0 AF364902				(Rousseaux *et al.*, 2001)
Pseudomonas sp.	NRRL B-12227	pLG221	Municipal sewage, Switzerland	99.0 AF312304						(Cook and Hütter, 1881; Eaton and Karns, 1991; Seffernick *et al.*, 2001)
Arthrobacter aurescens	TC1	pAA1	Agricultural soils, USA		100.0 AY456696	100.0 AY456696				(Strong *et al.*, 2002; Sajjaphan *et al.*, 2004)
Nocardioides sp.	SP12	nd	Experimental field, France		100.0 AF537330	100.0 AF537329				(Piutti *et al.*, 2003)
Arthrobacter crystallopoiete	Cit2	nd	Agricultural soils, France		99.1 AF364904	100.0 AF364905				(Rousseaux *et al.*, 2001)
Arthrobacter sp.	MCMB-436	**	Agricultural soils, India		98.2 AY589016	99.7 AY589013	99.8 AY594331			(Vaishampayan *et al.*, 2007)

*DNA sequence length; ** atz gene not detected on plasmids; nd: not determined.

Congugation of catabolic genes

To assess the mobility of the *atz* genes of strain MHP41, a conjugation experiment was performed. The strain MHP41 was conjugated with *P. putida* strain KT2442 and the presence of the catabolic *atz* genes in transconjugants was analyzed by PCR. The *atzD*, *atzE* and *atzF* genes of strain MHP41 were noted to be transferred by conjugation to strain *P. putida* KT2442 (Fig. 5), suggesting that these genes of strain MHP41 are mobile and, probably, located on a plasmid. The presence of the *atzD*, *atzE* and *atzF* genes in a plasmid has been observed in other bacteria such as *Pseudomonas* sp. ADP (Martínez *et al.*, 2001) and preliminary experiments observed *atzA* and *atzD* to be located on plasmid DNA, by southern hybridization, using labelled probes (data not shown).

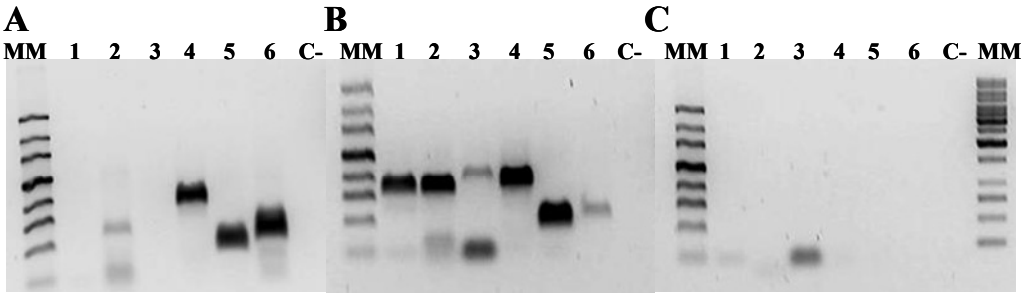

Figure 5. Mobility of *atz* genes from strain MHP41 to *P. putida* KT2442. Detection of *atzA, atzB, atzC, atzD, atzE* and *atzF* genes by PCR in a transconjugant strain. A. Transconjugant strain. B. *Pseudomonas nitroreducens* MHP41. C. *Pseudomonas putida* KT2442. MM. Molecular Marker (Multiple Choice Quantitative). *atzA* (lane 1), *atzB* (lane 2); *atzC* (lane 3); *atzD* (lane 4); *atzE* (lane 5); *atzF* (lane 6). Negative control (C).

Conclusions

In this work, we report the identification of strain MHP41 by the use of extensive phenotypic characterisation, 16S rRNA gene sequence analyses, IGS-1 sequence analyses and comparative analyses of house-keeping genes *gyrB* and *rpoB*. All analyses supported the conclusion that strain MHP41, which was isolated from Chilean soil and is an interesting candidate for the bioremediation of *s*-triazine, is a strain of the species *P.*

nitroreducens. Strain MHP41 represents the first example of *P. nitroreducens* reported to degrade s-triazine herbicides.

Experimental procedure

MHP41 growth and simazine degradation

Pseudomonas sp. MHP41 was isolated by enrichment from a long-term simazine-treated agricultural soil of central Chile (Hernández *et al.*, 2008b). Strain MHP41 has been deposited in the Culture Collection University of Gothenburg (CCUG), with the accession number CCUG 50378. *Pseudomonas* sp. MHP41 was grown in AM broth (Hernández *et al.*, 2008b), supplemented with simazine (0.5 mM) as the sole nitrogen source, and glucose as the sole carbon source. Cell growth was determined by measuring the turbidity at 600 nm. AM agar supplemented with simazine (3.0 mM) was used for cultivation on solid medium. Reference strains of *Pseudomonas* species were obtained from the CCUG and were cultivated on triptic soy agar (TSA) or blood agar media.

Exponential-phase cells grown in minimal medium using simazine as sole nitrogen source were harvested by centrifugation at 14,000 x g for 10 min, washed three times with a sodium phosphate (pH 7,0) and suspended in fresh buffer to a turbidity$_{600}$ of 0.5. Simazine was added to cell suspensions, to a final concentration of 0.06 mM. Cells were incubated at 30ºC. Aliquots of cell suspensions were taken at different incubation times. One volume of methanol (80% v/v, pH 2) was added to each aliquot and samples were centrifuged at 10,000 x g for 10 min to remove salts and other insoluble materials. High-performance liquid chromatography (HPLC) analysis was performed in a System Gold chromatograph (Beckman, Germany) equipped with a diode array detector and a RP-C18/Lichrospher 5-µm column (Supelco) as described previously (Morgante *et al.*, 2010). Degradation assays were performed in triplicate.

Taxonomic characterization and identification

Pseudomonas sp. MHP41 cells were grown to exponential phase in AM broth with simazine as the sole nitrogen source and glucose as sole carbon source. Cells were prepared and observed with a Zeiss EM900 transmission electron microscope as described (Cámara *et al.*, 2004; Agulló *et al.*, 2007).

Physiological and biochemical characterizations were performed, using API 20NE and API ZYM (BioMérieux, Marcy l'Etoile, France) and other typing assays (OX, OF, DEC, ESC and ASSIM), according to the worksheet and protocols of the CCUG Typing Lab for Gram-negative, aerobic non-fermenting bacteria (http://www.ccug.se/default.cfm?navID=160).

For genotypic-based analyses, genomic DNA was prepared, using the FastDNA® kit for bacteria and the protocols of the manufacturer (Bio 101 Systems, Q-BioGene). The 16S rRNA, gyrB (Yamamoto and Harayama, 1998) and rpoB (Tayeb *et al.*, 2005) genes and IGS-1 regions (Guasp *et al*., 2000) were amplified by PCR in 25 µl volume, in duplicate, using primers listed in Table S2 and the protocols described previously (Hauben *et al.*, 1997). Duplicate PCR-products were combined and checked by agarose gel electrophoresis and purified, using the QIAquick PCR Purification Kit and the protocols of the manufacturer (Qiagen, Hilden, Germany). Purified PCR-products were sequenced directly, using the primers listed in Table S2 and the *Taq* DyeDeoxy Terminator Cycle Sequencing kit, version 3.1, an ABI model 3100 DNA Sequencer and the protocols of the manufacturer (Applied Biosystems, Inc., Foster City, CA.). Sequence data were edited manually and compared to reference sequence data, including those of type strains of species with validly published names, using the FastA3 sequence matching tool (http://www.ebi.ac.uk/fasta3/) (Pearson and Lipman, 1988). Sequences were aligned and similarities were calculated, using the multiple alignment tool in the Kodon Bioinformatics package, version 3.0 (Applied Maths, BVBA, Sint-Martens-Latem, Belgium). Final alignments were completed by hand, using conserved sequence positions and secondary structure as references.

Sequence data of strain MHP41 and other strains analyzed were submitted to the EMBL Nucleotide Sequence Database (Kulikova *et al.*, 2007).

Table S2. Primer sets used in the present study

Primer	Sequence (5'−3')	Reference
atzA-F	CCATGTGAACCAGATCCT	(de Souza *et al.*, 1998)
atzA-R	TGAAGCGTCCACATTACC	
atzB-F	TCACCGGGGATGTCGCGGGC	
atzB-R	CTCTCCCGCATGGCATCGGG	
atzC-F	GCTCACATGCAGGTACTCCA	
atzC-R	GTACCATATCACCGTTTGCCA	
atzD-F	ACGCTCAGATAACGGAGA	(Fruchey *et al.*, 2003)
atzD-R	TGTCGGAGTCACTTAGCA	
atzE-F	GGTATCGCCTCTGGCAGAAC	(Cheng *et al.*, 2005)
atzE-R	GGCGATACCGGTGTCTTGT	
atzF-F	AAGATCTGGTCGAGTCAC	
atzF-R	TATTGAGCCGCGAGGTATGC	
rpoB		
LAPS-rpoB-F	TGGCCGAGAACCAGTTCCGCGT	(Tayeb *et al.*, 2005)
LAPS27-rpoB-R	CGGCTTCGTCCAGCTTGTTCAG	
gyrB external primers		
U-gyrB-F	CAYGSNGGNGGNAARTTYRA	(Yamamoto and Harayama, 1998)
U-gyrB-R	GCNGGRTCYTTYTCYTGRCA	
16S rRNA primers		
16F27	AGAGTTTGATCCTGGCTCAG	(Hauben *et al.*, 1997)
M16R518	CGTATTACCGCGGCTGCTGG	
16R1087	CTCGTTGCGGGACTTAACCC	
16R1494	TACGGYTACCTTGTTACGAC	
16F63	AGGCCTAACACATGCAAGTC	
M23R458	CCCCTTTCCCTCACGGTAC	(Guasp *et al.*, 2000)

Catabolic gene characterization

The *atzA, atzB, atzC, atzD, atzE and atzF* genes were amplified by PCR, using specific set of primers (Table S2). Sequence data were processed as described above and compared with reference sequence data.

Conjugation of catabolic genes

Strain MHP41 was used as donor strain in conjugation with the well-characterized recipient strain *P. putida* KT2442. Strain MHP41 is resistant to mercury (Hg), whereas strain KT2442 is resistant to rifampicin. Strain MHP41 was grown in LB medium supplemented with Hg (0.2 mM) and strain KT2442 was grown in LB broth medium supplemented with rifampicin (150 μg/ml). The mating experiments were performed in LB agar medium over night at 30ºC. For the selection of transconjugants, cells were grown in LB agar medium with mercury (0.2 mM) and rifampicin (150 μg/ml) and incubated over night at 30ºC. Transconjugants were analyzed by colony PCR to detect the presence of the *atz* genes as described previously by Hernández et al. (2008b).

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Chapter 5

Short-term effects of the herbicide simazine on microbial communities in agricultural soils

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Abstract

Simazine has been used worldwide as herbicide in agricultural soils. In this report, the short-term effects of simazine on *Bacteria, Fungi* and *Archaea* in agricultural soils from central Chile were analyzed. A long-term simazine-treated soil (soil A) and a soil that had not been previously exposed to the herbicide (soil B) were studied. Microbial community compositions of the soils were analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and 18S rRNA genes. Thereafter, predominant species represented in the DNA band profiles that were affected by simazine application were sequenced and identified. After simazine application in soils and short-term incubation (12 h), bacterial groups closely related to *Pseudomonas* and *Acinetobacter* genera were stimulated, irrespective of prior history of simazine application. Although fungal communities were only slightly affected by simazine application, this herbicide stimulated growth of fungal clones closely related to *Paecilomyces* and to *Penicillium* genera in soils with a prior history of simazine application. Additionally, simazine promoted changes in the composition of archaeal communities in both soils. The main shifts in the community composition involved members of the phylum *Crenarchaeote*. The present report indicates that short-term incubation with simazine promoted changes mainly in bacterial and archaeal community structures of agricultural soils, whereas the fungal community was slightly affected.

Introduction

The *s*-triazine herbicides are applied worldwide for the control of leaf and grassy weeds. Simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine) is one of the most used herbicides for agriculture and forestry in Chile, with an annual application of 219 tons (Hernández *et al.,* 2008a; Servicio Agrícola Ganadero, 2006). The *s*-triazines are endocrine disrupters and potential carcinogens (Birnbaum and Fenton, 2003; Hayes *et al.,* 2006). The large application of simazine in soils can potentially lead to contamination of water bodies. The environmental fate of simazine is determined by physico-chemical and biological processes (Barra-Caracciolo *et al.,* 2005; Flores *et al.,* 2009; Mandelbaum *et al.,* 1995; Morgante *et al.,* 2010). Diverse soil bacterial isolates are able to degrade these herbicides (Hernández *et al.,* 2008a; Hernández *et al.,* 2008b; Mandelbaum *et al.,* 1995; Rousseaux *et*

al., 2001; Topp *et al.,* 2000a; Topp *et al.,* 2000b) and the bioremediation of *s*-triazine-polluted soils has been studied (Morgante *et al.,* 2010; Rousseaux *et al.,* 2001).

Only a minor proportion of soil microbial communities are cultivable (Nakatsu *et al.,* 2000; Torsvik *et al.,* 1990). Therefore, a variety of culture-independent molecular methods, such as DGGE and fluorescent *in situ* hybridization (FISH), have been used for monitoring the spatial and temporal changes of soil microbial communities (Barra-Caracciolo *et al.,* 2005; Morgante *et al.,* 2010). These molecular profiling techniques have particularly useful application for studying the effect of pollutants on microbial communities.

The aim of this report was to study the short-term effects of simazine on the structure of bacterial, fungal and archaeal communities of agricultural soils from central Chile. The community 16S rRNA and 18S rRNA gene profiles were analyzed by DGGE and broad level changes in microbial compositions were detected. The predominant species of *Bacteria, Fungi* and *Archaea* represented in the DNA band profiles that were affected by simazine application were sequenced and identified.

Methods

Sample collection and DNA extraction

Soil samples (0-20 cm depth) were collected from agricultural sites located in the Quillota province, Aconcagua valley, central Chile (Flores *et al.,* 2009; Hernández *et al.,* 2008b). One soil was sampled from an avocado plantation (soil A) that had been treated annually with simazine for more than 20 years (2.5-4.0 Kg ha^{-1}). A second soil was sampled from a closely located orchard (soil B) with no prior history of simazine application (Flores *et al.,* 2009; Morgante *et al.,* 2010). Three independent soil samples were randomly collected from the surface layer (0–20 cm depth) in each plot, sieved, air-dried and stored at 4 °C. The physicochemical properties of soil samples used in this study are shown in Table 1. Soil A is a loam soil with 8.5% organic matter (OM) content, whereas soil B is clay-loam soil with 3.5% OM content. One gram of each soil was incubated in a sterile tube with 25 ml of AM medium (Rousseaux *et al.,* 2001) containing simazine at a final concentration of 0.5 mM. The tubes (in triplicate) were incubated with agitation for 12 h at 30 °C to culture the microbial populations (He *et al.,* 2005). Before and

after incubation with simazine, DNA was extracted from 0.5 g of soil samples using an Ultraclean Soil DNA Isolation Kit (Mo–Bio Laboratories, Inc.) according to the manufacturer's instructions.

Table 1. Physico-chemical properties of agricultural soils

	Soil	
	A	B
Organic matter (%)	8.5	3.5
pH-H_2O	6.4	7.2
N (mg kg^{-1})	17.0	15.0
P (mg kg^{-1})	57.0	59.0
K (mg kg^{-1})	383.0	364.0
K ($cmol_{(+)}$ kg^{-1})	0.98	0.93
Na ($cmol_{(+)}$ kg^{-1})	0.06	0.05
Ca ($cmol_{(+)}$ kg^{-1})	13.5	13.0
Mg ($cmol_{(+)}$ kg^{-1})	2.5	2.9
Al saturation (%)[a]	0.06	0.06

[a] Calculated as =Al/cation exchange capacity [Σ (K, Ca, Mg, Na, and Al)]×100
A. Soil with a long-term history of simazine application; B. Soil with no prior history of simazine application

PCR amplification

The compositions of the soil microbial communities were analyzed by PCR-DGGE of 16S rRNA and 18S rRNA genes. Briefly, for analysis of bacterial communities, the PCR amplifications were conducted using touchdown PCR with the primer set EUBf933–GC/EUBr1387 as described by Iwamoto et al. (2000) (Table 2). Analyses of the fungal and archaeal communities was carried out using nested PCR with touchdown PCR for the first round of DNA amplification (final annealing at 55°C). Touchdown PCR was performed using the primer sets NS1/NS8 (White *et al.,* 1990) and 21f /958r (Bano *et al.,* 2004) for *Fungi* and *Archaea*, respectively (Table 2). The second round of the nested PCR was performed with the primer sets NS7−GC/F1Ra for *Fungi* (de Souza *et al.,* 2004) and ARCf344−GC/517r for *Archaea* (Bano *et al.,* 2004) (Table 2).

Table 2. Primer sets used in this study

Microorganism and primer	Sequence (5'−3')	Reference
Bacteria		
EUBf933−GC	GCACAAGCGGTGGAGCATGT G	(Iwamoto *et al.,* 2000)
EUBr1387	GCCCGGGAACGTATTCACCG	(Iwamoto *et al.,* 2000)
GC-clamp*	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG	(Iwamoto *et al.,* 2000)
Fungi		
NS1	GTAGTCATATGCTTGTCT C	(White *et al.,* 1990)
NS8	TCCGCAGGTTCACCTACGGA	(White *et al.,* 1990)
NS7−GC	GAGGCAATAACAGGTCTGTGATGC	(De Souza *et al.,* 2004)
F1Ra	CTTTTACTTCCTCTAAATGACC	(De Souza *et al.,* 2004)
GC-clamp*	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG	(De Souza *et al.,* 2004)
Archaea		
21f	TTCCGGTTGATCCYGCCGGA	(Bano *et al.,* 2004)
958r	YCCGGCGTTGAMTCCAATT	(Bano *et al.,* 2004)
ARC344f−GC	ACGGGGCGCAGCAGGCGCGA	(Bano *et al.,* 2004)
517r	ATTACCGCGGCTGCTGG	(Bano *et al.,* 2004)
GC-clamp*	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCC	(Bano *et al.,* 2004)

*The GC−clamp was attached to the 5'−end of the primer

Denaturing gradient gel electrophoresis (DGGE)

The DGGE analysis was performed using a Bio-Rad Dcode system (Bio–Rad Laboratories, Inc.). Twenty µL of PCR product was loaded onto a 6% (w/v) polyacrylamide gel with a 50 to 70% gradient (80% denaturant stock solution contained 5.6 M urea and 32% (v/v) formamide). The electrophoresis was run for 12 h at 100 V. The gels were stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and visualized on an UV transilluminator. The dominant bands in the DGGE gels were carefully excised with a razor blade and the DNA was eluted overnight at 4ºC in tubes containing 50 µl of sterile DNA-free water (Kawai *et al.,* 2004). The eluted DNA was re-amplified with the same set of primers and a subsample was run on a DGGE gel to confirm the product. The DNA products were sequenced by Macrogen, Inc. (Korea).

The 16S rRNA and 18S rRNA gene sequences of the clones were deposited in the GenBank Nucleotide Sequence Database under accession numbers GQ996194-GQ996219. These sequences were compared, using BLAST tools, with those present in GenBank database from NCBI. The neighbor-joining tree was constructed based on sequences of reference strains taken from NCBI database and by using ClustalX program.

Results and discussion

The effect of simazine on microbial communities was studied in agricultural soils with different physical properties and history of herbicide application. Soil A is a loam soil with an 8.5% OM content and a long history of *s*-triazine application, whereas soil B is a clay-loam soil with a 3.5% OM content and with no prior history of simazine application. Each soil was incubated for 12 h in AM medium containing simazine (0.5 mM) at 30 °C. Sorption of simazine was moderate and reversible in both soils (Flores *et al.,* 2009). In a first approach, the effect of simazine on bacterial community structures was studied. The DGGE analysis revealed that the incubation with simazine, promoted changes in the bacterial community structures in both agricultural soils (Fig. 1a). Moreover, similar bacterial groups were stimulated by simazine in long-term simazine-treated agricultural soil and in a soil with no prior history of simazine application. These results suggest that these agricultural soils, independent of the previous history of simazine application, may be a reservoir of simazine-degrading bacteria. The reproducibility of the DGGE profiles between triplicate samples was high. Eight different DGGE bands that were strongly induced by simazine in both soils were sequenced to identify the bacterial clones (Table 2). These bacteria were selected as they likely include species that were able to degrade simazine and to use it as nitrogen source. The incubation with simazine stimulated growth of bacterial phylogenetic groups closely related to *Pseudomonas* (bands 1, 2, 3 and 4) and *Acinetobacter* genera (bands 5, 6 and 7) (Table 3). These results are in agreement with previous studies describing *Pseudomonas* strains able to use *s*-triazines as sole nitrogen source for growth (Cook and Hütter, 1881; Hernández *et al.,* 2008b; Mandelbaum *et al.,* 1995). Recently, simazine degradation in agricultural soils by bioaugmentation with a *Pseudomonas* strain has been reported (Morgante *et al.,* 2010). Contribution of *Acinetobacter* genus in the degradation of *s*-triazines has been also described (Singh *et al.,* 2004). By FISH analysis, an increase in alphaproteobacteria by simazine application in these agricultural soils was observed after four weeks (Morgante *et al.,* 2010). Secondly, in this study the effect of simazine on fungal communities was analysed. The DGGE analysis of fungal communities is shown in Fig. 1b. Simazine promotes changes in fungal communities in soil with a long history of *s*-triazine application. In contrast, no significant changes in the fungal communities were observed after incubation with simazine in soils no

previously treated with the herbicide. Compared with bacterial community, a lower reproducibility of DGGE profiles between fungal soil triplicates was observed. DGGE fungal bands induced by simazine were analyzed by 18S rRNA gene sequencing (Table 3). In soils with a history of *s*-triazine application, incubation with simazine stimulated a fungus closely related to the *Paecilomyces* genus (bands 9 and 10) in the three samples. Another fungus related to *Penicillium* (band 11) was also stimulated after simazine incubation. In contrast, no changes in the fungal communities were observed in soils with no prior history of simazine application after incubation with simazine . Recently, a *s*-triazine-degrading *Penicillium* isolate has been described (Singh *et al.,* 2008). In addition, it has been reported a *Paecilomyces* strain able to degrade the nitroaromatic herbicide pendimethalin (Singh and Kulshrestha, 1991).

Finally, in this report the effect of simazine on archaeal communities was studied. The DGGE analysis of archaeal communities is illustrated in Fig. 1c. The incubation of simazine stimulated some phylogenetic groups of archaea (bands 18, 19 and 20) in soils with a history of simazine application. However, the DGGE pattern also showed that simazine reduced some archaeal groups (bands 13 and 17) in this soil. In soils without a history of simazine application, incubation with simazine also stimulated the growth of several archaea (bands 21-26). Table 3 showed that the archaeal sequences of the DGGE bands are related with uncultured *Archaea*. The multiple alignment analyses revealed that all sequences of these uncultured archaea aligned within phylum *Crenarchaeota* (Fig. 2). Non-thermophilic *Crenarchaeota* are common inhabitant of terrestrial ecosystems. It has been proposed that these *Crenarchaeota* are the most abundant ammonia-oxidizing prokaryotes in soils (Kent and Triplett, 2002; Leininger *et al.,* 2006; Ochsenreiter *et al.,* 2003; Simon *et al.,* 2000; Schleper *et al.,* 2005). Compared with the huge diversity of bacteria, the *Archaea* diversity in soils seemed to be restricted to a few specific lineages (Ochsenreiter *et al.,* 2003).

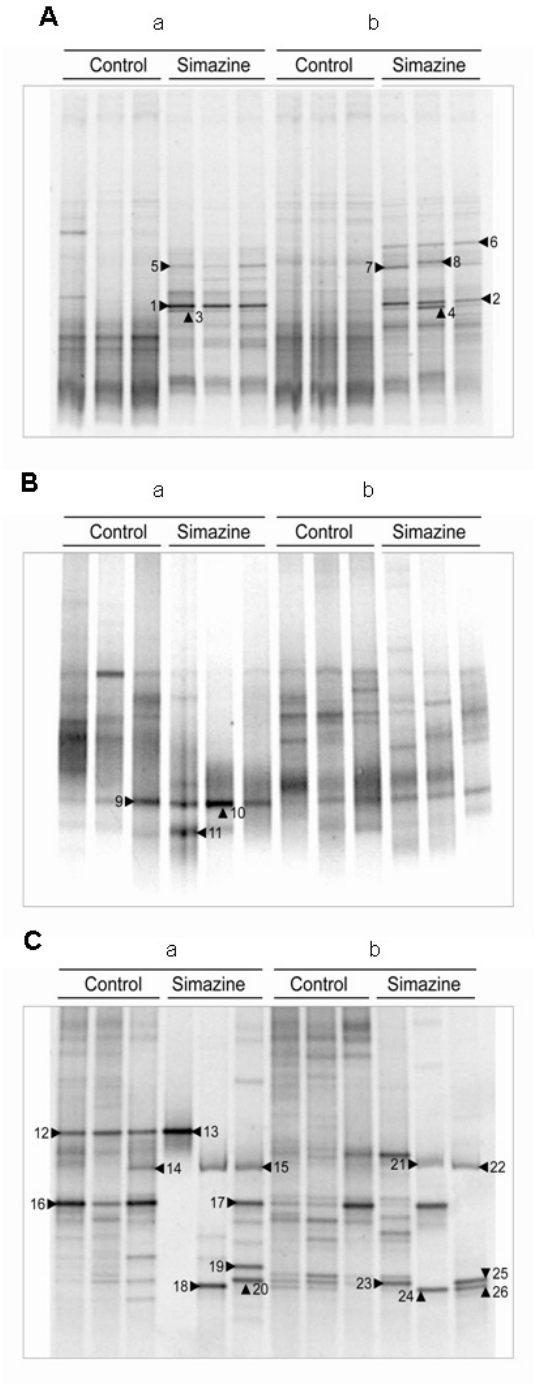

Figure 1. DGGE profiles of the microbial community composition in agricultural soils. Control, time 0 h. Simazine, after 12 h incubation with simazine 0.5 mM. a: soil samples with a long-term history of simazine application. b: soil samples with no prior history of simazine application. A. Bacterial community; B. Fungal community; C. Archaeal community. Arrows indicate the bands of interest that were sequenced.

Table 3. Phylogenetic assignment of DGGE bands

Band[a]	Taxonomic assigment[b]	Closest relative or cloned sequences (Accession number)	Similarity (%)[c]	Accession number
Bacteria				
1	*Gammaproteobacteria; Pseudomonas*	*Pseudomonas* sp. from perennial grass soil (AJ512403)	94	GQ996194
2	*Gammaproteobacteria; Pseudomonas*	*Pseudomonas* sp. from soil (FJ605433)	99	GQ996195
3	*Gammaproteobacteria; Pseudomonas*	*Pseudomonas fluorescens* (CP000094)	96	GQ996196
4	*Gammaproteobacteria; Pseudomonas*	*Pseudomonas* sp. from soil contaminated with polycyclic aromatic hydrocarbons (FJ668832)	98	GQ996197
5	*Gammaproteobacteria; Acinetobacter*	*Acinetobacter* sp. (GQ178058)	100	GQ996198
6	*Gammaproteobacteria; Acinetobacter*	*Acinetobacter* sp. from forest soil (DQ091245)	74	GQ996199
7	*Gammaproteobacteria; Acinetobacter*	*Acinetobacter* sp. from natural spring water (AM992174)	99	GQ996200
8	*Gammaproteobacteria; Pseudomonas*	*Pseudomonas fluorescens* from soil (GU059580)	99	GQ996201
Fungi				
9	*Ascomycota; Eurotiomycetes; Paecilomyces*	*Paecilomyces* sp. from mangrove (DQ401104)	99	GQ996202
10	*Ascomycota; Eurotiomycetes; Paecilomyces*	*Paecilomyces* sp. from manganese nodules in rice field subsoils (AB354568)	99	GQ996203
11	*Ascomycota; Eurotiomycetes Penicillium*	*Penicillium* sp. (AF245262)	82	GQ996204
Archaea				
12	*Crenarchaeota*	Uncultured crenarchaeote from soil (GQ127014)	90	GQ996205
13	*Crenarchaeota*	Uncultured crenarchaeote from river side soil (GQ906602)	89	GQ996206
14	*Crenarchaeota*	Uncultured crenarchaeote from sediment (EF090655)	89	GQ996207
15	*Crenarchaeota*	Uncultured crenarchaeote from heavy-metal-contaminated soil (GQ996208)	90	GQ996208
16	*Crenarchaeota*	Uncultured crenarchaeote from rhizosphere soil (EF021016)	94	GQ996209
17	*Crenarchaeota*	Uncultured crenarchaeote from shrubbery soil (GQ304804)	83	GQ996210
18	*Crenarchaeota*	Uncultured crenarchaeote from mineral soil (EF430844)	92	GQ996211
19	*Crenarchaeota*	Uncultured crenarchaeote from greenhouse soil (GU434687)	92	GQ996212
20	*Crenarchaeota*	Uncultured crenarchaeote from greenhouse soil (GU434680)	88	GQ996213
21	*Crenarchaeota*	Uncultured crenarchaeote from mineral soil (EF430734)	88	GQ996214
22	*Crenarchaeota*	Uncultured crenarchaeote from anoxic riverbank soil planted with rice (AM495414)	89	GQ996215
23	*Crenarchaeota*	Uncultured crenarchaeote from rhizosphere soil (EF021008)	93	GQ996216

24	*Crenarchaeota*	Uncultured crenarchaeote from mineral soil (EF430823)	89	GQ996217
25	*Crenarchaeota*	Uncultured crenarchaeote from mineral soil (EF430758)	91	GQ996218
26	*Crenarchaeota*	Uncultured crenarchaeote from soil (FJ957958)	94	GQ996219

[a] Corresponding DGGE bands shown in Fig. 1a, 1b and 1c

[b]The phylogenetic assignment is based on sequence analysis by the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) or GenBank database from NCBI (http://www.ncbi.nlm.nih.gov). It is given the phylum as well as the lowest predictable phylogenetic rank. [c]Based on partial sequencing of 16S rRNA and 18S rRNA genes and comparison with those present in GenBank by using Blastn.

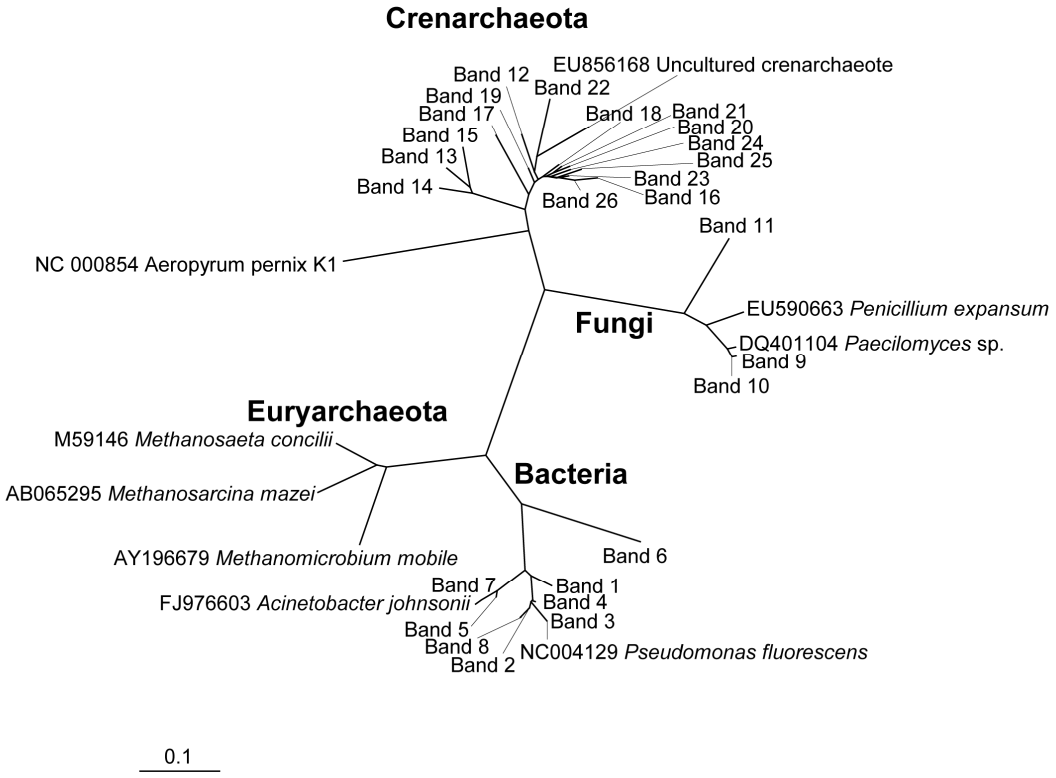

Figure 2. Unrooted phylogenetic tree of the *Bacteria*, *Archaea* and *Fungi* clones affected by simazine application in agricultural soils. Horizontal bar indicates 10% sequence divergence; bootstrap analysis was performed with 1,000 trials.

In conclusions, this study showed that the herbicide simazine stimulated the growth of gamma-proteobacteria in long-term simazine-treated agricultural soil and in a soil with no prior history of simazine application. Simazine stimulated bacteria closely related to *Pseudomonas* and *Acinetobacter* genera. The DGGE analysis also revealed that simazine promoted significant changes in archaeal communities in both soils. In contrast, simazine only slightly affected fungal communities in long-term simazine-treated agricultural. Thus, in these agricultural soils of central Chile, short-term incubation with the herbicide simazine promoted shifts in the community structures for all three phylogenetic groups.

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Chapter 6

Effect of simazine application on nitrification processes and the microbial communities in agricultural soil

Abstract

s-Triazine herbicides, such as simazine, has been used worldwide for weed control. *s*-Triazine herbicides persist in soils, as well as in aquifers. Nitrification, the biological oxidation of ammonia to nitrate, is of essential importance in the global nitrogen cycle. In the present study, the effect of simazine on the nitrification process by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) was studied by denaturing gradient gel electrophoresis (DGGE). Simazine, at a final concentration of 50 ppm blocks the nitrification in microcosm soils. DGGE patterns showed that AOB community is affected by the simazine incubation in the agricultural soil. However, no significant changes in the DGGE patterns by simazine in AOA community were found. These results obtained by DGGE analysis indicated that AOB community, rather than AOA community, is mainly affected by short-term simazine application in agricultural soil microcosms.

Introduction

Simazine is a *s*-triazine herbicide worldwidely used for the control of grass in a variety of agricultural and forestry soils. The toxicity of this *s*-triazine herbicide is of increasing concern. Despite their use has been forbidden in Germany since 1991(Tappe *et al.*, 2002), atrazine and simazine are still being used in some applications and are still detected in sea water, the highest levels are observed along the coast (United Nations Environment Programme Chemicals, 2002). Several strong inhibitors of NH_3 oxidation in soil can be classified by their heterocyclic ring structures (McCarty, 1999). Compounds containing two or three non-adjacent ring N atoms, such as pyrimide, *s*-triazine, benzimidazole can inhibit NH_3 oxidation in agricultural soils (McCarty and Bremner, 1989). Although simazine is a persistent pollutant, there is little information about the effects of simazine on the nitrification process (Debona and Audus, 1970; Gaur and Misra, 1977). Recently, the impact of the herbicide acetochlor in ammonia-oxidizing bacteria communities have been studied (Li *et al.*, 2008). In the recent years, substantial progress has been made towards understanding the modes of action for the specific inhibition of autotrophic NH_3 oxidation.

Nitrification involves the biological oxidation of ammonia to nitrate and is of fundamental importance in the global nitrogen cycle (Fig. 1) (Sahrawat, 2008; Shen *et al.*,

2008). The nitrification is strongly affected by physico-chemical parameters such as soil pH (Nicol *et al.*, 2008;), temperature (Avrahami *et al.*, 2003; Tourna *et al.*, 2008) and availability of CO_2 (Azam *et al.*, 2004).

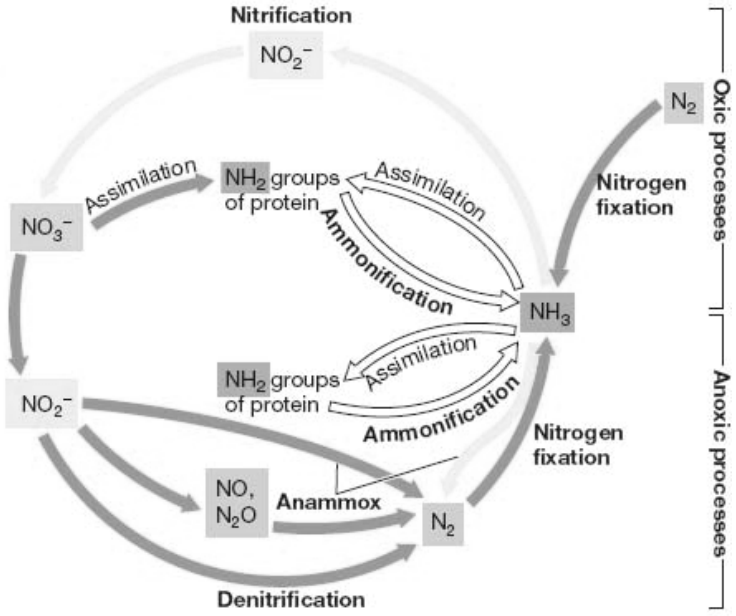

Figure 1. Redox cycle for nitrogen. Oxidation reactions are shown by light grey arrows, reductions reactions are shown by dark grey arrows. Reactions without redox change are in white arrows (modified from Madigan *et al*., 2009).

Bacteria able to grow chemolithotrophically at the expense of reduced inorganic nitrogen compounds are called nitrifying bacteria. These bacteria play key ecological roles in the nitrogen cycle, converting ammonia into nitrate, a key plant nutrient. Nitrifying bacteria are also important in sewage and wastewater treatment, where ammonia produced in the sediments from the decomposition of organic nitrogenous compounds is converted into nitrate for use by algae and cyanobacteria.

The key enzyme of ammonia oxidation is the ammonia monooxygenase. The α-subunit of the enzyme is encoded by the *amoA* gene, which can be used as a marker to identify these microorganisms in environmental samples (Chen *et al*., 2008). It was long time believed that microbial ammonia oxidation was solely performed by *Bacteria* and that only bacteria possess the *amoA* gene for the ammonia monooxigenase, recently, this

concept has been challenged, the *amoA* gene occurs in both Bacteria and in Archaea (Avrahami *et al*., 2003; Jia and Conrad, 2009; Leininger *et al.*, 2006; Nicol *et al.*, 2008; Shen *et al.*, 2008; Treusch *et al*., 2005). Quantitative analysis of the *amoA* genes showed that Archaea predominate among the ammonia-oxidizing prokaryotes in seawater (Karner *et al.*, 2001) and soil samples (Schleper *et al.*, 2005; Treusch *et al.*, 2005; Leininger *et al.*, 2006; Shen *et al.*, 2008).

Ammonia oxidizing bacteria (AOB) play an important role in the nitrogen cycle (Jia and Conrad, 2009; Shen *et al*., 2008). AOB involved in the aerobic oxidation of ammonia to nitrite are called Nitrosobacteria, which for example belong to the genus *Nitrosomas*. The bacteria responsible for the conversion of nitrite to nitrate are called Nitrobacteria, e.g. the genus *Nitrobacter* (Sahrawat, 2008). The accumulation of evidence for the existence and importance of mesophilic archaea in ammonia oxidation has had significant impact on the contemporary view of nitrification (Francis, 2007). Prosser and Nicol (2008) have reported that ammonia oxidizing archaea (AOA) are generally more abundant than the AOB, suggesting that archaea may play an important role in nitrification in many environments. Only a marine archaeon, *Nitrosopumilus*, has been isolated and characterized (Könneke *et al.*, 2005). *Nitrosopumilus* grows autotrophically with ammonia as the sole energy source and converts ammonia to nitrite with concomitant increase in cell number. The potential for crenarchaeal ammonia oxidation has also been confirmed by enrichment and isolation of ammonia-oxidizing archaea from hot springs (Hatzenpichler *et al*., 2008; de la Torre *et al*., 2008). However, the precise nature of the AOA has not been elucidated and to date there are no isolates from soil.

Fingerprints of AOB and AOA communities are often characterized by denaturing gradient gel electrophoresis (DGGE), which is an ideal tool for analyzing the sequence diversity of the complex natural microbial communities (Muyzer *et al.*, 1993; Spiegelman *et al*., 2005).

The aim of this work is to study the effect of simazine application on nitrification processes by colorimetric methods and on the structure of the microbial communities in soil by DGGE analysis.

Materials and methods

Soil sampling

Soils samples were collected from core down to 20-25 cm depth in triplicate in October 2009 from Brassicaeae plots located in an experimental station at the Giessen University in Rauischolzhausen, Germany. The soil was subjected to fertilization with 120 Kg ha^{-1} of urea and 50 Kg h^{-1} of a solution including ammonium, nitrate and urea. These soils samples, which have not had simazine application for more than 16 years, were air-dried, sieved (2 mm diameter) and stored in polyethylene bottles at 4 °C until analysed.

Preliminary experiments

To determine the optimal simazine concentrations of the sample soils, preliminary experiments were development. To determine optimal simazine concentration, batch microcosms were analysed. 10 g oven dry soil was incubated with two different simazine concentrations, 10 and 50 ppm and NH_4 at 15 ppm final concentration. As control, 100 Pa of acetylene (C_2H_2) was used (Bollmann and Conrad, 1997). All the experiments were done in triplicate.

Microcosms experiment

Ten grams (d.w.s) soil samples from 0-25 cm depth were pre-incubated for one week at 25% WHC in presence and/or absence of C_2H_2 (100 Pa) and simazine (10 ppm). Then, samples were sprayed with water, final concentrations used were 70 ppm of $(NH_4)_2SO_4$ and 50 ppm of simazine until obtain a soil moisture content of 60% water-holding capacity (WHC), incubated at 28ºC and darkness in 120 ml serum bottles sealed with butyl stoppers. 70 ppm of $(NH_4)_2SO_4$ was added every week to the correspondent treatment. Samples were performed in presence and/or absence of 100 Pa C_2H_2 as negative control. Destructive samples were carried out at zero time and every week until 4 weeks for chemical analysis and nucleic acid extraction. All the experiments were performed in triplicate.

Nitrification activity

Soil nitrate concentration was determined by colorimetric method. After incubation for 60 min at 4ºC with 1M KCl, the extracted soil samples were filter with a whatman paper and nitrate concentrations were determined by spectrophotometer at 430 nm (Hart *et al.*, 1994).

Denaturing gradient gel electrophoresis of *amoA* gene

The analysis of microbial community composition in the soils samples was evaluated by denaturing gradient gel electrophoresis (DGGE), after extraction of DNA from soils with a CTAB protocol described by Jia and Conrad (2009). The analysis of bacterial and archaeal *AmoA* gene were carried out by a normal PCR (30 cycles and annealing of 55°C for 1 min) followed by nested PCR (24 cycles and annealing of 55°C for 1 min). The initial PCR for bacterial *AmoA* gene was performed by using the primer set amoA-1F/2R (Rotthauwe *et al.*, 1997). For archaeal AmoA gene, PCR was carried out with the primers Amo19F/643R (Treusch *et al.*, 2005; Jia and Conrad, 2009). The nested PCR for bacteria and archaea were performed with the addition of a GC clamp in the primers amoA-1F and Amo643R respectively. DGGE for bacteria was performed using a denaturing gradient from 30% to 70% (urea and formamide) and for archaea was a denaturing gradient from 20% to 50% (urea and formamide). The DGGE analysis was performed using a DCode system (Bio–Rad Laboratories, Inc.). About 15−20 μL of PCR product was loaded onto a 6% (w/v) polyacrilamide gel with the correspondent gradient (urea and formamide). The electrophoresis was run in 1X Tris Acetate EDTA buffer for 16 h at 100 V at 60ºC. The gel was then stained with 1:10000 SYBR Gold I for 30 min and scanned with a Typhoon Trio variable Model Imager (Molecular Dynamics, Sunnyvale, CA).

Results

In the present work, we studied the effect of the herbicide simazine on the structure and function of ammonia oxidizing microorganisms from German agricultural soils. Due to previous studies according to evaluate the nitrification potential activity, the selected agricultural soil is useful to evaluate the effect of simazine on nitrification (Jia and Conrad, 2009). In a first approach, the oxidation of ammonia in the presence of different simazine concentrations in a non-treated agricultural soil was analyzed. Simazine at a concentration of 50 ppm is able to block the nitrification process (Fig. 2).

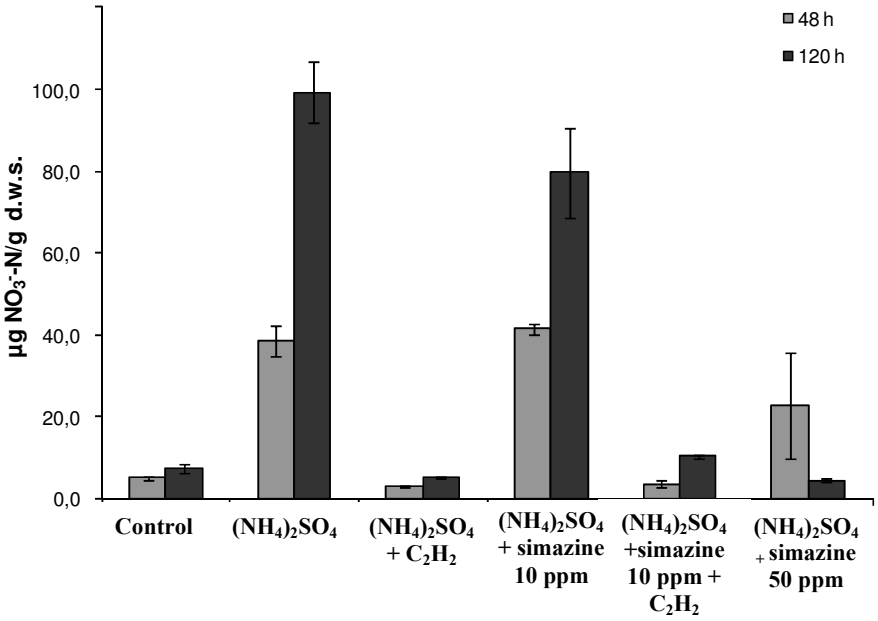

Figure 2. Effect of simazine concentrationon nitrıtıcatıon ın soil. Bars represent standard deviations of triplicate incubation.

Ammonium addition resulted in the accumulation of nitrate after three weeks incubation at 60% maximum water holding capacity (WHC). After four weeks, nitrate accumulation reaches 80%. Nitrification is inhibited by simazine immediately after one week of incubation. As we expected, addition of C_2H_2 at 100 Pa completely inhibits the nitrate production (Fig. 3).

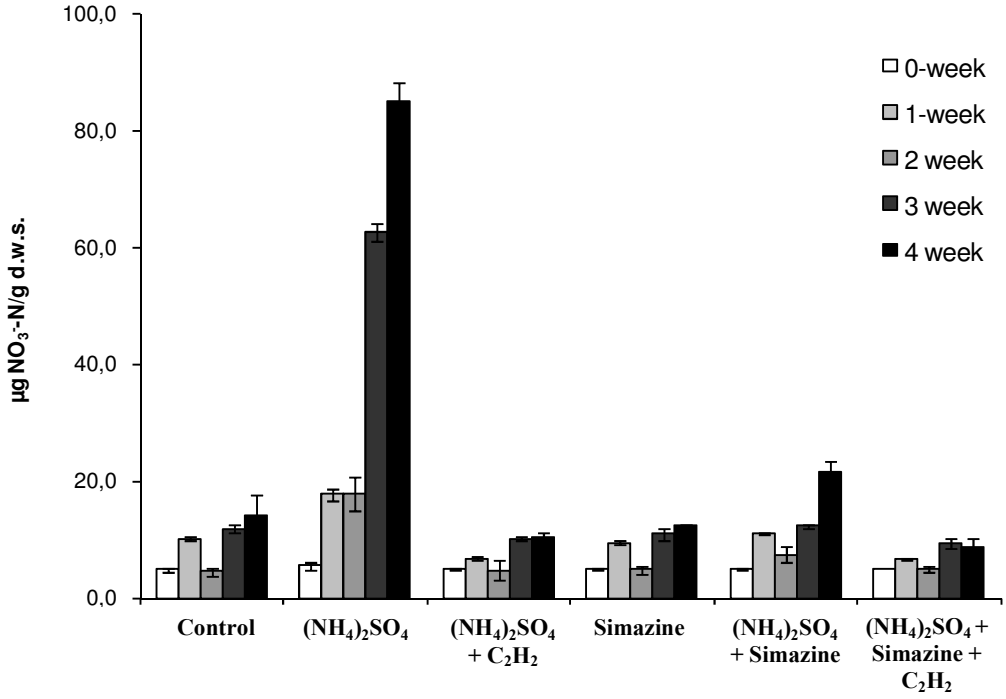

Figure 3. Effect of simazine on nitrification in agricultural soils Bars represent standard deviations of triplicate incubations.

The present study assessed the microbial community structure of ammonia oxidizers by targeting the *amoA* gene coding for ammonia monooxygenase analysed by denaturing gradient gel electrophoresis. The DGGE patterns for *amoA*-containing bacteria were affected by the different treatments. The relative intensity of DGGE band "a" and "b" was enhanced by addition of ammonium and inhibited by addition of simazine (Fig. 4). The comparison of this DGGE patterns with the DGGE patterns of the same soil studied by Jia and Conrad (2009), suggest that the DGGE bands "a" and "b" belong to *Nitrosospira* species, we were not able to elucidate the bands 1 to 7 in bacterial DGGE profile. The Figure 5 shows the archaeal DGGE fingerprinting. Simazine did not affect the DGGE patterns, no differences were observed in all the treatment during the four weeks of incubation. Bands 8 to 16 belong to the uncultured Crenarchaeota.

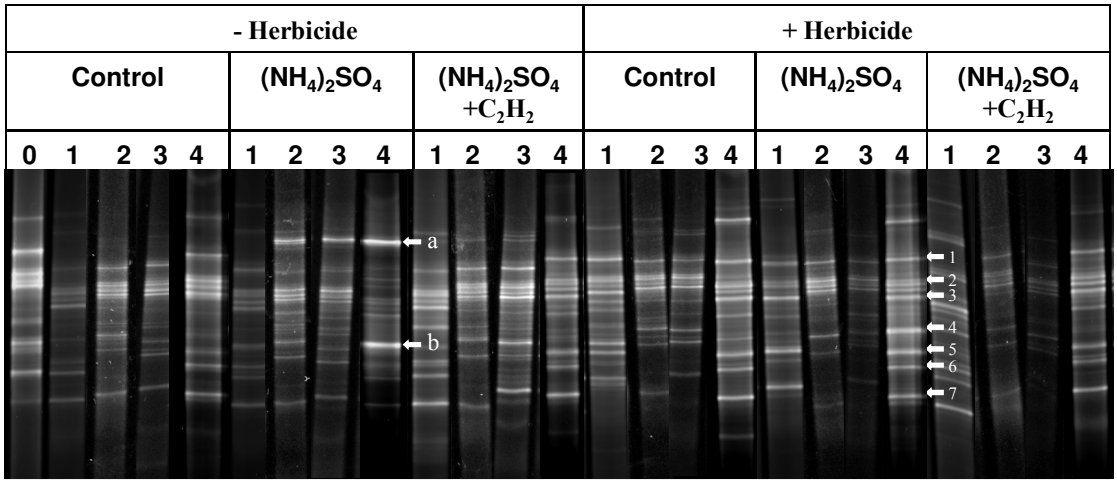

Figure 4. Effect of simazine on DGGE fingerprints of the composition of bacterial *amoA* genes in surface (0–20 cm depth) agricultural soil. Bands "a" and "b" could correspond to *Nitrosospira* species. Bands 1 to 7 not detected in the sequence analysis.

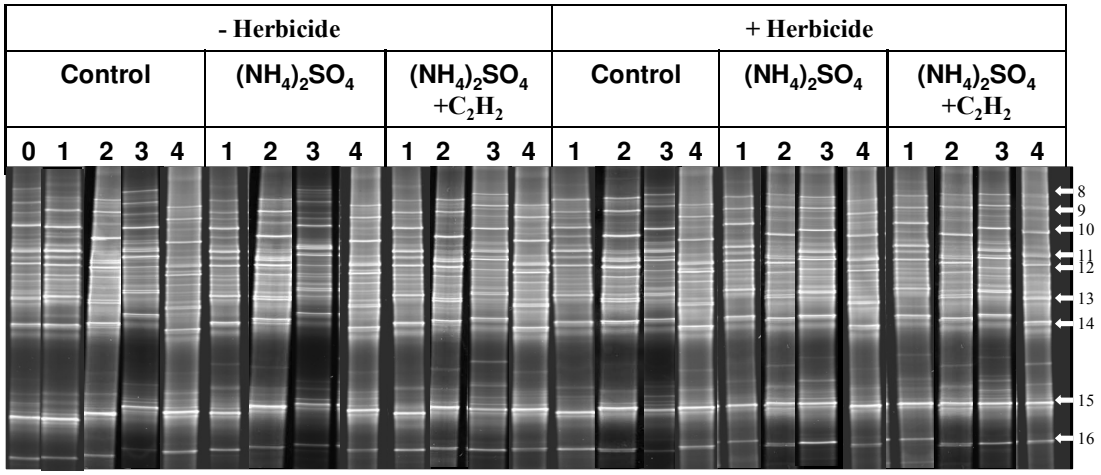

Figure 5. Effect of simazine on DGGE fingerprints of the composition of archaeal *amoA* genes in surface (0–20 cm depth) agricultural soil. Bands 8 to 16 belong to the uncultured Crenarchaeota.

Discussion

The nitrification potential is strongly affected by physico-chemical parameters such as soil pH (Nicol *et al.*, 2008), temperature (Avrahami *et al.*, 2003; Tourna *et al.*, 2008) and availability of CO_2 (Azam *et al.*, 2004). Heterocyclic N compounds form, another

important class of nitrification inhibitors with little known about their inhibitory influence is closely related to the presence of ring N. In the present study, 5-times field-doses of simazine recommended for Chilean agricultural soils, strongly affected the nitrification. Is previous studies, compounds containing two or three non-adjacent ring N atoms, such as pyrimide, *s*-triazine, benzimidazole had effect on NH_3 oxidation in agricultural soils treated with 0.1 μmol g^{-1} of each compound (McCarty and Bremner, 1989). The herbicide simazine is also an important parameter that can affect the nitrification process.

In these microcosms study, in which ammonia oxidation is stimulated by ammonium and inhibited by acetylene, the herbicide *s*-triazine simazine causes a strong effect on nitrification production. Although these soils are not in contact with the herbicide simazine for a long period, once inoculated with this, the nitrification production is affected. This is the fist report who studied the effect of simazine on nitrification in the last 30 years. Gaur and Misra (1977) have studied the effect of simazine, lindane and ceresan up to 100-fold of field application on the nitrification rates, and they found that simazine up to 20 ppm initially hampered the nitrification rate, but at the day 35 it was comparable with the control. However, that study revealed that simazine over 200 ppm was toxic through the entire period. The present study also revealed that 50 ppm of simazine is an enough concentration to observe variations on the nitrification rate.

On the other hand, the present work is an attractive study how shows that archaea are probably not so much involved in ammonia oxidation as postulated recently (Leininger *et al.*, 2006; Chen *et al.*, 2008). It is possible that only the fertilizer-induced rates of ammonia oxidation but not the background rates were due to bacterial rather than archaeal activity. A recent study developed by Jia and Conrad (2009) revealed that the bands enhanced by ammonium inoculation belong to the nitrifying bacterium *Nitrosospira*. These results suggest that this type of species could be affected by simazine application. Indeed it was observed that candidatus '*Nitrososphaera gargensis*' isolated from hot spring was inhibited by relatively high concentrations of ammonium (Hatzenpichler *et al.*, 2008). The oxidation of ammonia into nitrite is also performed by the nitrifying bacteria *Nitrosomas* and *Nitrobacter* (Xia *et al.*, 2005; Sahrawat, 2008; Shen *et al.*, 2008). However, it should be noted that oxidation of fertilizer ammonium makes up the most substantial part of total

ammonia oxidation (Gruber and Galloway, 2008) and that this part is dominated by bacterial ammonia oxidizers in agricultural soil as suggested by current study.

Finally, the present study indicated that simazine had a significant effect on the activity of ammonia oxidation and on the structure of *amoA*-containing bacteria community, but not on the community structure of *amoA*-containing archaea in the agricultural soil studied.

Acknowledgments

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Global discussion

Simazine is a *s*-triazine herbicide worldwide used for agriculture and forestry. The *s*-triazines atrazine, simazine and cyanazine have been classified by EPA as a possible human carcinogen of class C. To minimize the damages produced by these herbicides, processes for the removal of *s*-triazines from the polluted sites have been studied. The main mechanism for the removal of these compounds from the environment is the microbial degradation. Diverse bacteria and mixed communities of microorganisms able to degrade *s*-triazine have been isolated. The genes *atzA, atzB, atzC, atzD, atzE* and *atzF* are involved in the *s*-triazine degradation pathways (see appendix book chapter).

In the present thesis, culture-dependent and culture-independent methods were used for the analyses of the microorganisms of agricultural soils. In the first part, *s*-triazine degrading strains were characterized and identified. The catabolic *atzA, atzB, atzC, atzD, atzE* and *atzF* genes were determined. On the other hand, we employed the culture-independent method denaturing gradient gel electrophoresis (DGGE) to evaluate the effect of the herbicide simazine on the *Bacteria, Fungi* and *Archaea* in agricultural soils and to analyse the influence of simazine on the nitrification process and the ammonia-oxidizing bacteria (AOB) and the ammonia-oxidizing archaea (AOA) communities.

Diverse simazine-degrading strains isolated from Chilean agricultural soils were characterized and identified. These strains were identified by a variety of multi locus sequence analysis as *Arthrobacter* (by 16S rRNA gene sequence analysis), *Stenotrophomonas* (by 16S rRNA gene and *gyrB*, and *rpoD* housekeeping genes sequence analysis) and *Pseudomonas nitroreducens* (by 16S rRNA, *gyrB* and *rpoB* housekeeping genes sequence analysis, as well as 16S-23S rRNA inter-genic spacer sequence analysis).

The results obtained in the identification of the *Stenotrophomonas* sp. strains in the present work suggest that these strains probably comprise a new species. Multi locus sequence analysis (MLSA) is an alternative, more flexible way of comparing bacteria, towards the development of a species concept. Analyses of these conserved protein-encoding housekeeping genes sequences provide higher resolution than the analysis of 16S rRNA gene sequences.

In the present work, the strain *Pseudomonas nitroreducens* strain MHP41 was extensively characterized. This novel *s*-triazine-degrading bacterium efficiently degrades simazine, is capable of growing fast using simazine as the sole source of nitrogen and

yielding high biomass. *P. nitroreducens* sp. MHP41 possesses all *atz* genes of the upper and lower catabolic pathways for simazine degradation. *P. nitroreducens* sp. MHP41 has a biotechnological potential for the bioremediation of *s*-triazine contaminated soils (see appendix patent).

On the other hand, this study showed that the herbicide simazine stimulated the growth of gamma-proteobacteria in long-term simazine-treated agricultural soil and in a soil with no prior history of simazine application. Simazine stimulated bacteria closely related to *Pseudomonas* and *Acinetobacter* genera. The DGGE analysis also revealed that simazine promoted significant changes in archaeal communities in both soils. In contrast, simazine only slightly affected fungal communities in long-term simazine-treated agricultural. Thus, in these agricultural soils of central Chile, short-term incubation with the herbicide simazine promoted shifts in the community structures for all three phylogenetic groups. Finally, the present thesis indicated that simazine had a significant effect on the activity of nitrification process and on the structure of *amoA*-containing bacteria community, but not on the community structure of *amoA*-containing archaea in an agricultural soil.

Outlook

The process of biodegradation of *s*-triazines is still not well understood and its microbial and molecular basis has to be further studied.

Strain MHP41 and the other strains characterized in this thesis could be potential biocatalysts for bioremediation processes to remove *s*-triazines from polluted environments. Further studies of these simazine-degrading bacteria are required to analyze their potential to bioremediate soils contaminated with *s*-triazine herbicides.

The *s*-triazine catabolic pathways in bacteria are still under evolution. In the strain *Pseudomonas* sp. ADP it has been shown that only the *atzD, atzE* and *atzF* are clustered in an operon and are regulated by a transcriptional regulator and by nitrogen sources (see appendix book chapter). The catabolic pathway gene organization and regulation of the *s*-triazine-degrading bacterial strains should be determined.

Mobile genetic elements play a primary role in the development and dissemination of catabolic genes and allowed bacterial populations to rapidly adapt to a strong selective pressure. The presence of the *atz* genes in plasmids in the *s*-triazine-degrading bacterial strains have to be further studied to evaluate the contribution of these mobile genetic elements in the bacterial adaptability and diversity.

General Conclusions

- Diverse bacterial strains isolated from agricultural soils of central Chile and able to degrade simazine were characterized by microbiological and biochemical tests and by 16S rRNA and housekeeping gene sequence analyses. Bacterial strains were identified as species of *Pseudomonas*, *Stenotrophomonas* and *Arthrobacter*.

- The bacterial strains isolated from agricultural soils from central Chile possess *atz* genes encoding enzymes of *s*-triazine catabolic pathways.

- *Pseudomonas nitroreducens* strain MHP41 is a bacterium strain isolated from Chilean agricultural soil from Quillota valley (Chile) and able to growth using simazine as only nitrogen source and to degrade efficiently simazine. Strain MHP41 possesses the six *atz* genes that codified the enzymes for the upper and lower catabolic simazine pathways. *Pseudomonas nitroreducens* MHP41 is an efficient biocatalyst for the bioremediation of simazine polluted soils. Strain MHP41 represents the first strain of *Pseudomonas nitroreducens* able to degrade the *s*-triazine herbicides.

- The application of simazine in agricultural soils affects the community structure of bacteria and archaea. The herbicide simazine stimulated bacteria of the genus *Pseudomonas* and *Acinetobacter* and archaeas of the phylum Crenarchaeote in agricultural soils. After incubation with simazine, slight changes in fungal communities in agricultural soils were observed. Simazine stimulated fungi closely associated with *Paecilomyces* and *Penicillium* genera.

- The application of simazine in agricultural soils inhibited the nitrification activity and caused changes in community structure of ammonia-oxidizing bacteria (AOB), but not in the community structure of ammonia-oxidizing archaea (AOA).

Conclusiones Generales

- Diversas cepas bacterianas aisladas desde suelos agrícolas de Chile central y capaces de degradar herbicidas se caracterizaron por pruebas microbiológicas y bioquímicas y por análisis de las secuencias del gen 16S rRNA y de genes de manutención celular. Las bacterias fueron identificadas como especies de *Pseudomonas*, *Stenotrophomonas* y *Arthrobacter.*

- *Pseudomonas nitroreducens* MHP41 es una cepa bacteriana aislada desde suelos agrícolas del valle de Quillota (Chile), que tiene la capacidad de crecer utilizando simazina como única fuente de nitrógeno y de degradar eficientemente simazina y que posee los seis genes *atz* que codifican las enzimas de las rutas superior e inferior de degradación de simazina. *Pseudomonas nitroreducens* MHP41 es un eficiente biocatalizador para la biorremediación del herbicida *s*-triazina. La cepa MHP41 representa la primera cepa de la especie *Pseudomonas nitroreducens* capaz de degradar los herbicidas de la familia de las *s*-triazinas.

- Las cepas aisladas desde suelos agrícolas de Chile central poseen genes catabólicos *atz* que codifican las enzimas de las rutas catabólicas de *s*-triazinas.

- La aplicación de simazina en suelos agrícolas afecta la estructura de las comunidades de bacterias y arqueas. El herbicida simazina estimuló bacterias de los géneros *Pseudomonas* y *Acinetobacter* y arqueas del phylum Crenarqueas en suelos agrícolas. Después de la incubación con simazina, se observaron ligeros cambios en las comunidades de hongos en suelos agrícolas. Simazina estimuló hongos estrechamente asociados a los géneros *Paecilomyces* y *Penicillium*.

- La aplicación de simazina en suelos agrícolas inhibió la actividad de nitrificación y provocó cambios en la estructura de las comunidades de bacterias amonio-oxidasas (AOB), pero no en la estructura de las comunidades de arqueas amonio-oxidasas (AOA).

Appendix

Organization of the thesis

The organization of the present thesis consists mainly of a general introduction, three published papers, two submitted papers and two manuscripts in preparation.
They will be referred to in the text of the thesis as follow:

Chapter 1: General introduction

Chapter 2: Modern approaches for the study of bioremediation of *s*-triazine herbicides in agricultural soils
Hernández, M., V. Morgante, C. Flores, P. Villalobos, M. González, P. Miralles, M. A. Dinamarca and M. Seeger. 2008. Journal of Soil Science and Plant Nutrition 8: 19-30.

Chapter 3, Part I: Novel *s*-triazine-degrading bacteria isolated from agricultural soils of central Chile for herbicide bioremediation
Hernández, M., V. Morgante, M. Ávila, P. Moralles, M. González, and M. Seeger. 2008. Electronic Journal of Biotechnology 11: 01-07.

Chapter 3, Part II: Molecular characterization and phylogeny of novel herbicide-degrading *Stenotrophomonas* sp. strains isolated from Chilean agricultural soil
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Chapter 5: Short-term effects of the herbicide simazine on microbial communities in agricultural soils

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Chapter 6: Effect of simazine application on nitrification processes and the microbial communities in agricultural soil

Hernández, M., Z. Jia, R, Conrad, and M. Seeger. 2010. Manuscript in preparation.

Contributions of co-authors

All manuscripts published, submitted or in preparation for publication were written by Marcela Hernández with the support of the co-authors, especially from Dra. Verónica Morgante, Dr. Milko Jorquera, Dr. Edward Moore and Dr. Michael Seeger.

Molecular studies and data analyses were performed by myself. MLSA were advised by Edward Moore (Chapters 3 and 4). The HPLC measurements in Chapters 3 and 4 were performed by Myriam González. DGGE analyses were made by myself with kind support of Dr. Milko Jorquera and Dr. Zhongjun Jia (Chapters 5 and 6). The design of field experiment was supervised by Dra. Verónica Morgante in Chapter 5 and in Chapter 6 was supervised by Dr. Ralf Conrad and Dr. Zhongjun Jia.

Further publications, book chapter and patent

Publications

Martínez, P., L. Agulló, **M. Hernández,** and M. Seeger. 2007. Chlorobenzoate inhibits growth and induces stress proteins in the PCB-degrading bacterium *Burkholderia xenovorans* LB400. Arch. Microbiol. 188: 289-297.

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Book chapter

Seeger, M., Morgante, V. and **Hernandez, M.** 2010. Bacterial degradation of herbicides in soils. In: Microbial Populations: Structure and Evolution (Lodeiro, A., ed.), Vol 1, Editorial Research Signpost, Kerala, India, In press.

Patent

Hernández, M., Morgante, V., Villalobos, P., Flores, C., González, M. and Seeger, M. 2007-2008. s-Triazine herbicide-degrading bacteria, product for the bioremediation and method of bioremediation. Chile Patent, No 1982-2007 dated 06.07.2007. USA Patent, No 12/166,961 dated 02.07.08. Canada Patent, No 2,636,856 dated 07.07.08. Colombia Patent, No 0806 8859 dated 04.07.08. Argentina Patent, No 08 01 02903 dated 04.07.09.

Explora-Conicyt activities

Explora is a national education program based on science and technology created by the National Commission of Investigation, Science and Technology (Conicyt), Chile. The main objective of Explora is to contribute to the creation of a scientific and technological culture in the community, particularly for those in scholar age, through non-formal educational activities in order to develop the ability to appropriate the benefits of these areas.

Explora activities and talks were developed in the following educational establishments:

Actividades de Explora-Conicyt

Explora es un programa nacional de educación basada en la ciencia y la tecnología creada por la Comisión Nacional de Investigación, Científica y Tecnología (Conicyt) de Chile. El principal objetivo de Explora es contribuir a la creación de una cultura científica y tecnológica en la comunidad, particularmente en quienes se encuentran en edad escolar, mediante acciones de educación no formal, con el propósito de fortalecer la apropiación de los beneficios de la ciencia y la tecnología.

Actividades de Explora y charlas fueron realizadas en los siguientes establecimientos educacionales:

2006. XII Semana Nacional de la Ciencia y la Tecnología. Universidad de La Frontera, Temuco, Chile. Explora activity.

2007. Empleo de bacterias para la remediación del medio ambiente. XIII Semana Nacional de la Ciencia y la Tecnología. 1000 CIENTIFICOS, 1000 AULAS. Talk in Liceo de Ciencias y Humanidades de Pitrufquén, Pitrufquén, Chile.

2009. Biorremediación de suelos impactados por pesticidas. Proyecto: Descubriendo el trabajo de los organismos microscópicos mediante la fabricación de compost: desde la microbiología a la biotecnología. Talk in Liceo Los Guindos de Buin and Liceo Politécnico de Buin, Santiago, Chile.

2009. Biodegradación de herbicidas en Chile. XV Semana Nacional de la Ciencia y la Tecnología. 1000 CIENTIFICOS, 1000 AULAS. Talk in Colegio Manantial, Viña del Mar, Chile.

2010. Scientific adviser of research in Science and Technology developed by children and youth of the region. School Congress of Science and Technology. Valparaíso, Chile.

Appendix

Book Chapter

Transworld Research Network
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Bacterial Populations: Basic and Applied Aspects of Their Structure and Evolution, 2010:000-000
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6. Bacterial degradation of *s*-triazine herbicides in soils

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Abstract. Pesticides are extensively used for the agriculture and forestry. The herbicides are the most applied agrochemicals. *s*-Triazines are herbicides worldwide used since more than forty years for the control of weeds in soils. The toxicity and the wide distribution of *s*-triazines such as atrazine and simazine are of increasing environmental concern. For a sustainable development, the use of pesticides has to be regulated and the fate of pesticides in the environment has to be controlled. Retention and mobility of a pesticide in soil are governed by sorption processes. Organic matter and clay minerals are main sorbents of *s*-triazines in soils. Biological degradation of pesticides is the main mechanism for removal of these compounds from the environment. Diverse bacteria capable to degrade *s*-triazines have been isolated from soils. Recently, an efficient *s*-triazine-degrading strain, *Pseudomonas* sp. strain MHP41, was characterized. The metabolism of *s*-triazines has been elucidated in some bacterial strains. The upper catabolic *s*-triazine pathway converts simazine or atrazine into cyanuric acid, whereas

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the lower pathway mineralizes cyanuric acid. The catabolic *atz* and *trz* genes encode the enzymes of the upper and lower pathways. The *s*-triazine catabolic gene sequences and organization have been described in few bacterial strains. For the removal of herbicides from the environment, bioremediation is an attractive technology due to its *in situ* application, the low cost, the mineralization of the pollutants and the minimal environmental disturbance. Biostimulation and bioaugmentation are the main bioremediation techniques. Bioaugmentation of *s*-triazine-polluted soils has been reported. However, only few herbicide bioremediation studies have been successful. The selection of the bacterial strain is critical for the bioremediation. Culture-independent methods such as FISH, DGGE and T-RFLP have been useful to analyze the biodiversity and to characterize the structure and function of microbial communities exposed to herbicides and involved in bioremediation. Finally, research needs to understand bacterial degradation of herbicides and to design bioremediation processes are described.

Introduction

The use of pesticides has been important for the development of the agriculture and forestry in the last half century increasing strongly the productivity. The pesticides are employed to control weeds, insects and pathogenic microorganisms. However, the usage of pesticides is of increasing concern due to their toxicity, persistence and widely distribution.

The most abundant agrochemicals used worldwide are the herbicides (Figure 1) [1]. The United States is the main herbicide consumer worldwide, with an application of around 205,000 tons annually [2], whereas the European Union registered an annual herbicide consumption of around 130,000 tons [3]. In South America, the annual application of herbicides was around 75,000 tons [1]. In this developing region, the main herbicide consumers are Brazil (38%), Argentina (28%), Colombia (18%) and Chile (7%) (Figure 2).

For a sustainable development, pesticide usage has to be controlled and the fate of the pesticide residues has to be managed. Biological degradation of pesticides is the main mechanism for removal of these compounds from the environment. Therefore, bioremediation is an attractive technology for the decontamination of pesticide-polluted sites. Bioremediation is based on the degradation activity of native or introduced microorganisms in the polluted sites.

The study of microbial communities in a herbicide-contaminated soil is crucial to understand the herbicide biodegradation and to design bioremediation processes. The integration of culture-dependent and culture-independent methods enables monitoring the microbial communities inhabiting the ecosystems as well as the microorganisms responsible for pesticide catabolism.

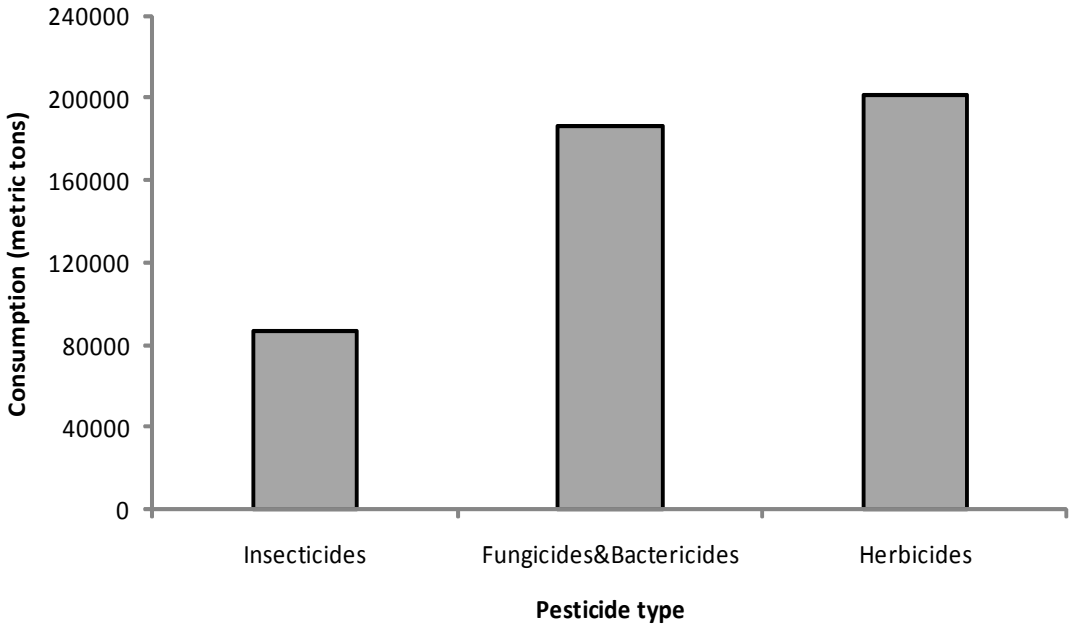


Figure 1. World pesticide consumption in 2001. Values were obtained and adapted from the Statistical Division of the Food and Agriculture Organization of the United Nations [1].

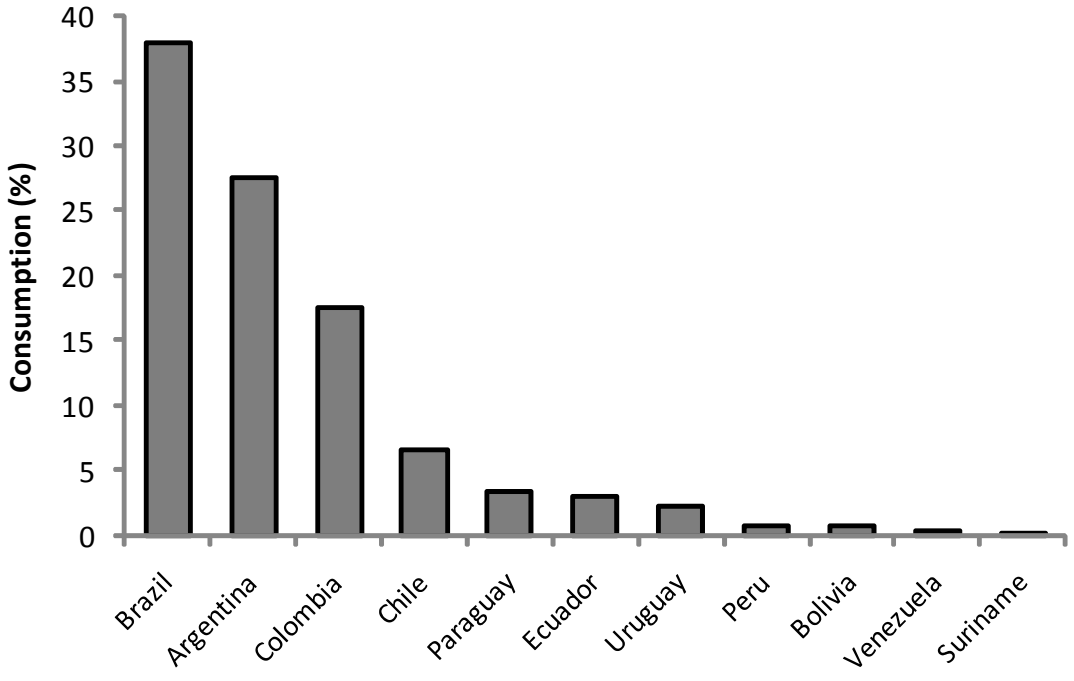
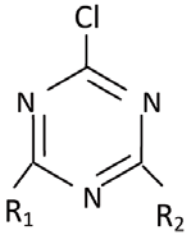


Figure 2. Herbicide consumption in South America from 1990 to 2001. Values were adapted from the Statistical Division of the Food and Agriculture Organization of the United Nations [1].

s-Triazine herbicides

s-Triazines are herbicides extensively used since more than forty years for the control of weeds in agriculture, forestry and non crop soils in many regions of the world [4, 5, 6]. *s*-Triazines are applied as pre-and post-emergence herbicides in diverse plantations such as maize, sorghum, alfalfa, citrus, grape, avocado and olive-fields. The *s*-triazines are strong inhibitors of photosynthesis in susceptible plants, interrupting the electron transport chain in photosystem II [7]. Atrazine and simazine are main members of the *s*-triazine herbicides (Figure 3).

The toxicity, persistence and the wide distribution of *s*-triazines in the environment are of increasing concern [6]. *s*-Triazines act as endocrine disrupters and impaired amphibian sexual development [8]. These herbicides altered development of amphibians and mammals [9, 10, 11]. Atrazine caused mammary cancer in rats and has been classified as a class C carcinogen [9, 12, 13]. After application only a minor fraction (<0.1%) of a pesticide reaches the target pest, whereas the main part enters the environment as a pollutant [14]. Simazine is the second most commonly detected pesticide in surface and groundwater in the United States, Europe and Australia [13, 15, 16].



***s*-Triazine**	**R_1**	**R_2**
Atrazine	$NHCH_2CH_3$	$NHCH(CH_3)_2$
Cianazine	$NHC(CN)(CH_3)_2$	$NHCH(CH_3)_2$
Propazine	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
Simazine	$NHCH_2CH_3$	$NHCH_2CH_3$

Figure 3. Chemical structures of *s*-triazines. Members of *s*-triazine herbicides differ by their R_1 and R_2 groups around the *s*-triazine ring.

Due to their toxicity, the use and the environmental concentration of *s*-triazines have been regulated in many countries. The maximum pesticide acceptable levels of *s*-triazine herbicides in drinking water is 0.1 µg l^{-1} for Europe and is 3.0 µg l^{-1} for USA [17, 18]. In 2001, the European Union includes atrazine and simazine in a list of "priority hazardous substances" [19]. In 2004, *s*-triazines have been banned in many European countries [19]. However, in North and Latin America there are still no restrictions for their use [3].

Environmental fate of *s*-triazines in soil

Once the herbicide has been applied, many abiotic and biotic processes may influence the fate of the herbicide in the environment (Figure 4).

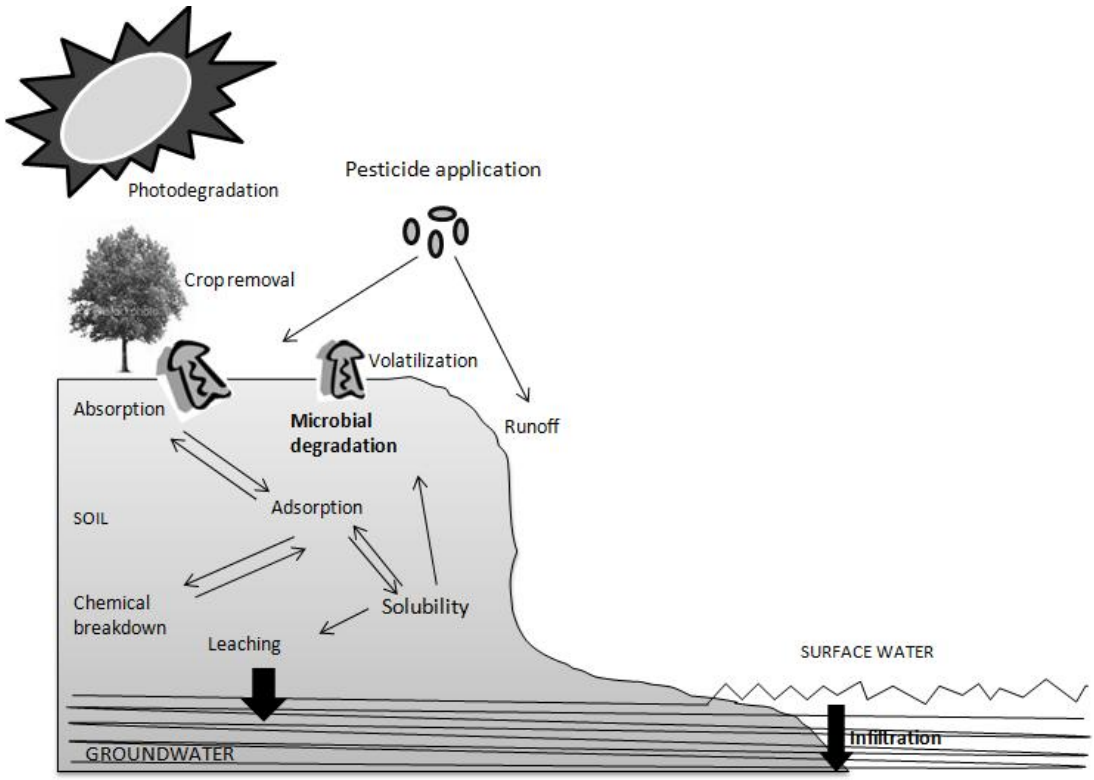


Figure 4. Environmental fate of herbicides. An herbicide applied into soil can undergo a number of fundamental processes. The absorption is the uptake of the herbicide by plants and microorganisms. The adsorption involves the binding of the herbicide to soil particles. Transport of pesticides within the soil compartment can occur downward into the soil profile (leaching), across the soil surface (runoff), or into the air (volatilization). Degradation processes include biological degradation by soil microorganisms, chemical breakdown and photodegradation.

Sorption of the herbicide to soil components reduces its bioavailability. Some processes could transport the herbicide away from the target plant, while other processes degrade the herbicide. Volatilization, plant uptake, leaching and runoff transport the herbicide from an application site. Degradation processes of the herbicide include photolysis, microbial degradation and chemical transformation.

The persistence of herbicides in soil is determined by several factors. Soil physical and chemical properties such as texture, organic matter content as well as the pH strongly influence herbicide half life. In addition, chemical properties of the herbicide such as solubility, vapor pressure, and the susceptibility to chemical or microbial degradation determine the environmental stability of the compound. Climatic variables such as moisture and temperature are also relevant factors. However, the metabolic activities of soil microorganisms play a central role on herbicide fate in soil.

Retention and mobility of a pesticide in soil are governed by sorption processes. Sorption is determined by physical-chemical properties of the soil and the pesticide [20]. Sorption interactions of pesticides in the soil may involve the mineral and organic components [21, 22]. In soils with low organic matter content, pesticide adsorption has been associated with the active components of the inorganic fraction, predominantly the clay. For soils with high organic matter levels (>5%), pesticide retention depends on binding to the organic matter [23]. For *s*-triazines, soil organic matter and clay minerals are main sorbents [22, 24].

Selection of herbicide-degrading bacterial strains

In the 1980s Cook *et al.* [25-27] isolated *Pseudomonas* and *Klebsiella* strains able to utilize *s*-triazines as the sole nitrogen sources for growth. Interestingly, two of these isolates, *Pseudomonas* sp. strains D and F, were able to use *N*-ethylammelide, *N*-isopropylammelide and cyanuric acid as sole nitrogen source for growth. *N*-ethylammelide, *N*-isopropylammelide and cyanuric are metabolic intermediates of atrazine and simazine degradation. Since this pioneer study, many bacteria capable to degrade *s*-triazines have been isolated. However, only few of these isolated microorganisms have been selected for metabolic studies and as biocatalysts for bioremediation processes to remove *s*-triazines from polluted environments (Figure 5). Isolation of bacteria by enrichment using the *s*-triazine as sole nitrogen source has been the most used selection procedure. *Pseudomonas* sp. strain ADP was isolated from a soil exposed to herbicide spills and is able to use atrazine as the sole nitrogen source for growth [17]. Strain ADP has been the model bacterium for the study of degradation of *s*-triazines and its atrazine metabolism has been extensively

characterized [28]. Diverse Gram-negative and Gram-positive bacteria, predominantly *Pseudomonas*, *Arthrobacter*, *Chelatobacter*, *Agrobacterium*, *Pseudaminobacter* and *Nocardiodes* strains, capable to degrade *s*-triazine herbicides have been isolated [3, 7, 17, 18, 29-34]. Recently, bacterial strains able to degrade and use simazine as the sole nitrogen source for growth were isolated from agricultural soils in central Chile [3, 34]. These isolates were identified as *Pseudomonas*, *Rodococcus*, *Stenotrophomonas* and *Arthrobacter* species. An efficient *s*-triazine-degrading strain, *Pseudomonas* sp. strain MHP41, was further characterized (Figure 6) [34].

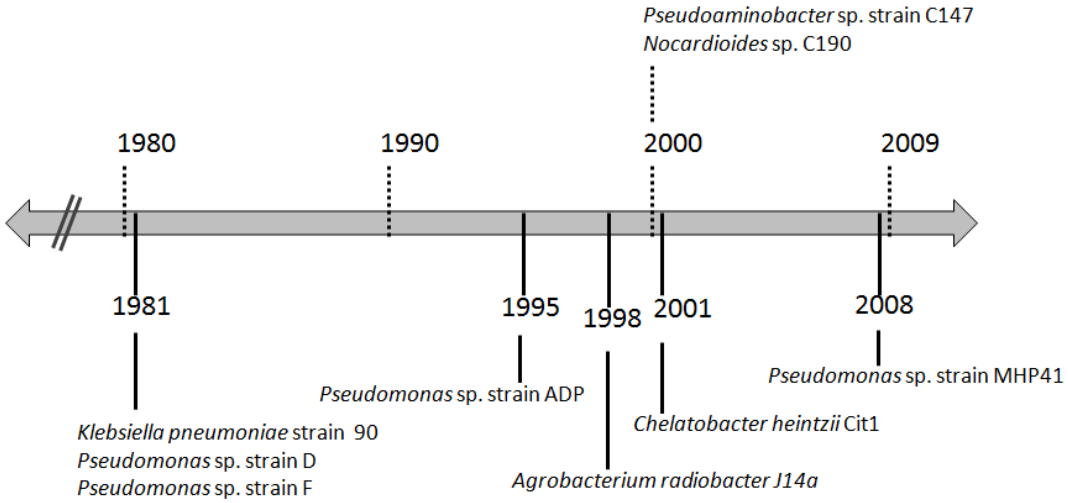


Figure 5. History of the isolation of *s*-triazine-degrading bacteria used for metabolic and bioremediation studies.

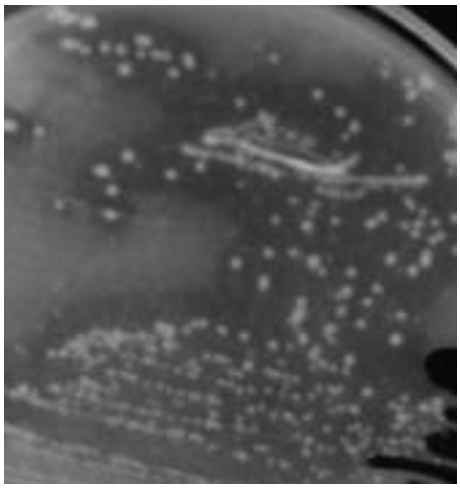


Figure 6. Simazine degradation by *Pseudomonas* sp. MHP41. Bacteria were grown on minimal medium agar plates supplemented with simazine as sole nitrogen source. Clearing zones around colonies indicate simazine degradation by bacteria.

Metabolic pathways and genes for *s*-triazine degradation

The metabolism of *s*-triazine in bacteria has been elucidated (Figure 7). These herbicides are degraded by an upper and a lower catabolic pathway. The upper catabolic pathway for *s*-triazines converts simazine or atrazine into cyanuric acid (Figure 7A). These enzymes of the upper degradation pathway are encoded by the *atzA, atzB* and *atzC* genes [35]. The enzyme atrazine chlorohydrolase AtzA catalyzes the hydrolytic dechlorination of simazine to yield hydroxysimazine, which is further transformed through deamination into *N*-etilammelide and *N*-etilamine by the AtzB hydrolase. Finally, *N*-etilammelide is hydrolyzed by AtzC hydrolase producing cyanuric acid and a second molecule of *N*-etilamine. A different initial hydrolase for *s*-triazine herbicide catabolism, TrzN, which has broader substrate specificity than AtzA has been described mainly in Gram-positive bacteria (*Arthrobacter aurescens* TC1, *Nocardioides* sp. C190, *Nocardioides* sp. SP12) [31, 36-38], but also later in Gram-negative bacteria such as *Sinorhizobium* and *Polaromonas* strains [39].

The lower pathway for *s*-triazines mineralizes cyanuric acid in three steps (Figure 7B) [28]. The three enzymes of the lower degradation pathway are encoded by the *atzD, atzE* and *atzF* genes [7]. Cyanuric acid is converted by cyanuric acid amidohydrolase AtzD into biuret, which is further transformed by biuret hydrolase AtzE into allophanate. The third reaction of the lower pathway converts allophanate by the enzyme allophanate hydrolase AtzF into carbon dioxide and NH_3. A *trzD* gene coding an enzyme responsible for the *s*-triazine ring cleavage of cyanuric acid has also been described [18, 39, 40]. The cyanuric acid amidohydrolase TrzD is inhibited by barbituric acid [40]. The *trzD* gene was found in different *s*-triazine-degrading bacteria such as *Pseudomonas, Chelatobacter, Aminobacter, Acidovorax, Klebsiella, Alcaligenes* and *Ralstonia* strains [18, 39-41].

The *s*-triazine catabolic gene sequences and organization was first reported in *Pseudomonas* sp. ADP. Strain ADP possesses the complete atrazine degradation pathway encoded by the *atzA, atzB, atzC, atzD, atzE* and *atzF* genes. These genes are located on a plasmid (Figure 8) [28]. The *atz* genes of the upper pathway are distributed in the plasmid of strain ADP. The *atzDEF* genes of the lower pathway of strain ADP are clustered in an operon and are regulated by a transcriptional regulator AtzR [42]. The expression of the *atzDEF* operon is regulated by nitrogen sources. The *atzDEF* genes are similarly organized in an operon in *Agrobacterium* sp. NEA-D [39]. Relatively few *s*-triazine-degrading strains that posses the six genes encoding the enzymes for mineralization of these herbicides have been described

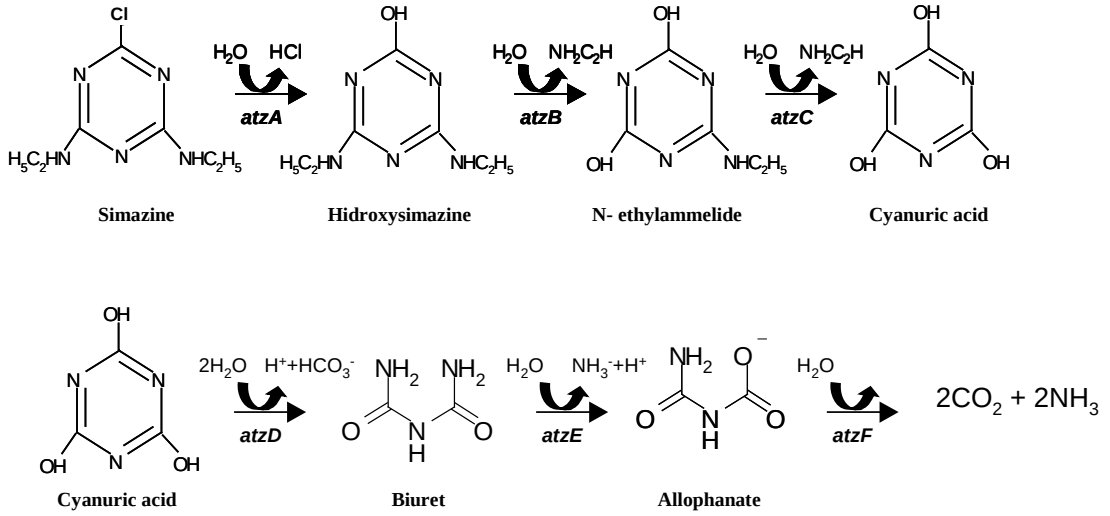


Figure 7. The simazine catabolic pathways. A, the upper catabolic pathway. B, the lower catabolic pathway. The catabolic *atz* gene encoding the respective enzyme is indicated at each metabolic step.

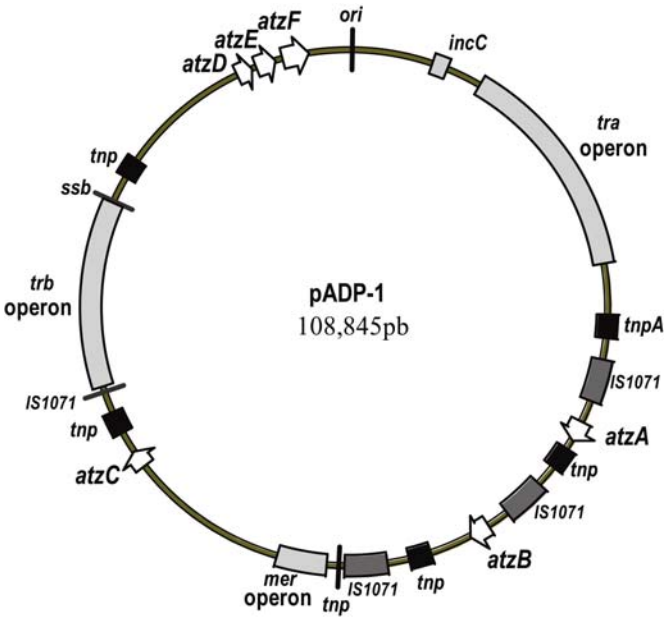


Figure 8. The catabolic plasmid pADP-1 containing the atz genes encoding the enzymes for atrazine degradation in Pseudomonas sp. ADP. Adapted from Martínez *et al.* [28].

[17, 34, 39]. *Pseudomonas* sp. MHP41 possesses the *s*-triazine catabolic *atzA, atzB, atzC, atzD, atzE* and *atzF* genes (Figure 9) [34]. The *atz* genes are highly conserved among a taxonomic range of bacteria. Other bacteria possess a different catabolic gene organization. The *atzABCtrzD* gene

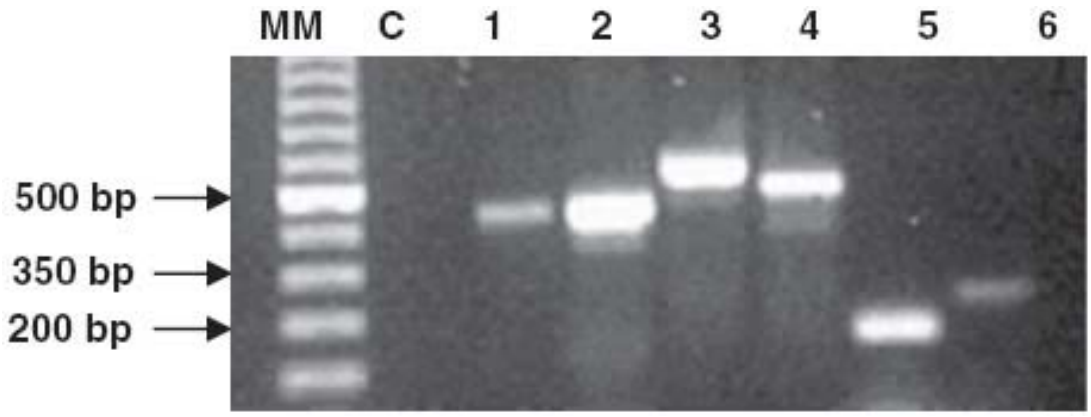


Figure 9. Detection of a*tz* catabolic genes for simazine degradation in *Pseudomonas* sp. MHP41. PCR-amplification products: *atzA* (lane 1), *atzB* (lane 2); *atzC* (lane 3); *atzD* (lane 4); *atzE* (lane 5); *atzF* (lane 6). MM: molecular mass marker (Roche, Basel); C: negative control. Adapted from Hernández *et al*., 2008 [34].

combination was present in most of the *Chelatobacter heintzii* strains [18, 39]. A relationship between the presence of the *atzABCDEF* or *atzABCtrzD* gene combinations and the mineralization capabilities of bacterial strains has been described [18, 39]. Some bacteria have only few *atz* genes. Only *atzB* and *atzC* genes were detected in some *Arthrobacter* strains [7, 18]. Only the *atzA* gene was detected in some *Chelatobacter heintzii* isolates and in *Stenotrophomonas maltophilia* Lous3-4 [18]. The *atz* and *trz* genes were often located on plasmids and associated to transposons, suggesting that horizontal gene transfer is important in their dispersion. As diverse *s*-triazine degrading microbes have several catabolic *atz* and *trz* combinations, these are probably recently evolved genes.

Bioremediation of herbicides in soils

For the removal of persistent organic pollutants from the environment, bioremediation is often more attractive than conventional physicochemical technologies. Some advantages of bioremediation are the *in situ* application, the low cost, the complete degradation or mineralization of the pollutants and the minimal environmental disturbance. Bioremediation uses living organisms, primarily microorganisms (e.g. bacteria, fungi), to remove the contaminants from the polluted environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and introduced to the contaminated site.

Natural attenuation is a process carried out by the indigenous microorganisms that inhabit the polluted site. The contaminant is degraded by

native microorganisms through their metabolism. The monitoring of natural attenuation is one of the bioremediation techniques. However, frequently the indigenous microbes are not efficient in the removal of the pollutants and this process is very slow.

The main bioremediation techniques are biostimulation and bioaugmentation. Biostimulation is a technology that adds nutrients to the polluted sites, to stimulate the native microbiota and their degradation capabilities. Bioaugmentation involves the addition to the contaminated environment of degrading microorganisms, which will increase the degradation rate in the environment.

The persistence of *s*-triazines in different polluted sites suggests a low natural microbial degradation potential. In these sites, the addition of microorganism is required to increase the catabolic potential. Bioaugmentation by the addition of bacteria for the removal of *s*-triazines in soil has been described [30, 43-47]. However, many failure associated with bioaugmentation have been reported [48-51].

The success of bioaugmentation depends on several factors such as the environmental conditions (e.g. moisture, nutrients, redox, pH and osmotic factors), the microbial ecology (e.g. indigenous microbiota competition, predation, grazing) and on the use of the appropriate bacterial strains. The selection of the bacterial strain is critical for the bioremediation [51]. A strain with high pesticide degrading capabilities and fast growth rate is required [52, 53]. In addition, the adaptation to the soil of the inoculated strain and a long term survival are important. Native bacterial strains often show higher bioremediation efficiency than exogenous bacteria [47, 54].

The inoculum density is also critical for the bioremediation. The strain has to be inoculated at high density into the polluted site. A high inoculum is useful to overcome in soil, competition with native bacteria, predation by protozoa and bacteriophage and low pollutant bioavailability due to sorption [52]. Different inoculum densities have been applied for removal of atrazine in soils [46, 54]. Additionally, the inoculation system is an essential factor in bioaugmentation. Inoculation of cells immobilized in polymeric materials is an interesting strategy. Encapsulation (for example, in alginate) protects bacteria from predators and nutrient stress, preserves long term cell viability and permits exchange of nutrients and excretion products. Repeated applications of the strain overcome long-term survival problems [44].

Bioaugmentation of *s*-triazine-polluted soils has been reported [30, 43- 47, 54]. The inoculation of a bacterial consortium of three strains increased mineralization of atrazine in in nonplanted and planted soil [43]. The addition of *Agrobacterium radiobacter* J14a increased from two to five times atrazine degradation in soil non-adapted to this herbicide [30].

Bioaugmentation with *Chelatobacter heintzii* Cit1 of soils non-adapted to atrazine increased *s*-triazine mineralization [54]. The bioremediation by inoculation of *Pseudaminobacter* sp. strain C147 and *Nocardioides* sp. strain C190 accelerated atrazine removal in soil [46]. On the other hand, *Pseudomonas* sp. strain ADP, failed to accelerate atrazine degradation in soil (~40 to 300 mg kg^{-1} of atrazine) [46, 55]. However, repeated inoculation of *Pseudomonas* sp. strain ADP removed atrazine from a contaminated soil [44]. In addition, bioaugmentation with *Pseudomonas* sp. strain ADP combined with biostimulation removed atrazine in a highly polluted soil [56]. Significant increased biotransformation of atrazine in a highly polluted site was observed using chemically killed recombinant *Escherichia coli* engineered to overproduce atrazine chlorohyrolase AtzA [45]. Recently, successful bioaugmentation with *Pseudomonas* sp. strain MHP41 of simazine in agricultural soils was reported [47]. Noteworthy, *Pseudomonas* sp. strain MHP41 was essential for the simazine removal in highly contaminated soil.

Microbial communities of soils treated with herbicides

Microorganisms play key functions in geochemical cycles in soil. For the fate of herbicides in soil the microbial communities are crucial. Microorganisms are involved in herbicide degradation and are the main catalysts for bioremediation processes. The cultivation of soil microorganisms has been very useful to study the microbial metabolism, genetics and physiology. However, only a small fraction of the soil microorganisms are yet cultivable. Isolated microorganisms are estimated to constitute less than 1% of all microbial species of the soil [57, 58]. The study of the structure of the autochthonous microbial communities and their dynamics helps to understand the effects of exogenously added microorganisms on the microbial community composition.

Soils that have been long term treated with *s*-triazines have generally a higher herbicide-degradation activity. Application of high doses of atrazine in soil increased microbial biomass, soil microbial respiration and some enzymatic activities [5, 59]. Recently, a simple and sensitive method for the detection and enumeration of *s*-triazine-degrading microorganisms in soil has been reported [5]. It is based on the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) to detect metabolic activity and the most-probable-number (MPN) enumeration in microtiter plates. This technique detects the capability of the microbes to use a *s*-triazine herbicide as sole nitrogen source. The MPN-TTC method has been useful to observe an increase of simazine-degrading and cyanuric acid-degrading microorganisms

in agricultural soil after herbicide application [5]. However, culture-dependent techniques such as the MPN-TTC method underestimate the degradation potential of microbial communities in soil.

The development of culture-independent methods enables characterizing the structure and function of microbial communities in soil (Figure 10). The molecular biology tools allow circumventing the requirement for cultivation in the characterization of the microbial diversity in an environment. The structure of microbial communities could be studied by the analysis of specific gene sequences. The 16S ribosomal RNA gene sequence analysis is generally used for studying the diversity of microbial communities in the environment. The 16S rRNA is a crucial component of the small subunit of the ribosome for the biosynthesis of polypeptides. The 16S rRNA gene sequence analysis of the microbial community by molecular approaches such

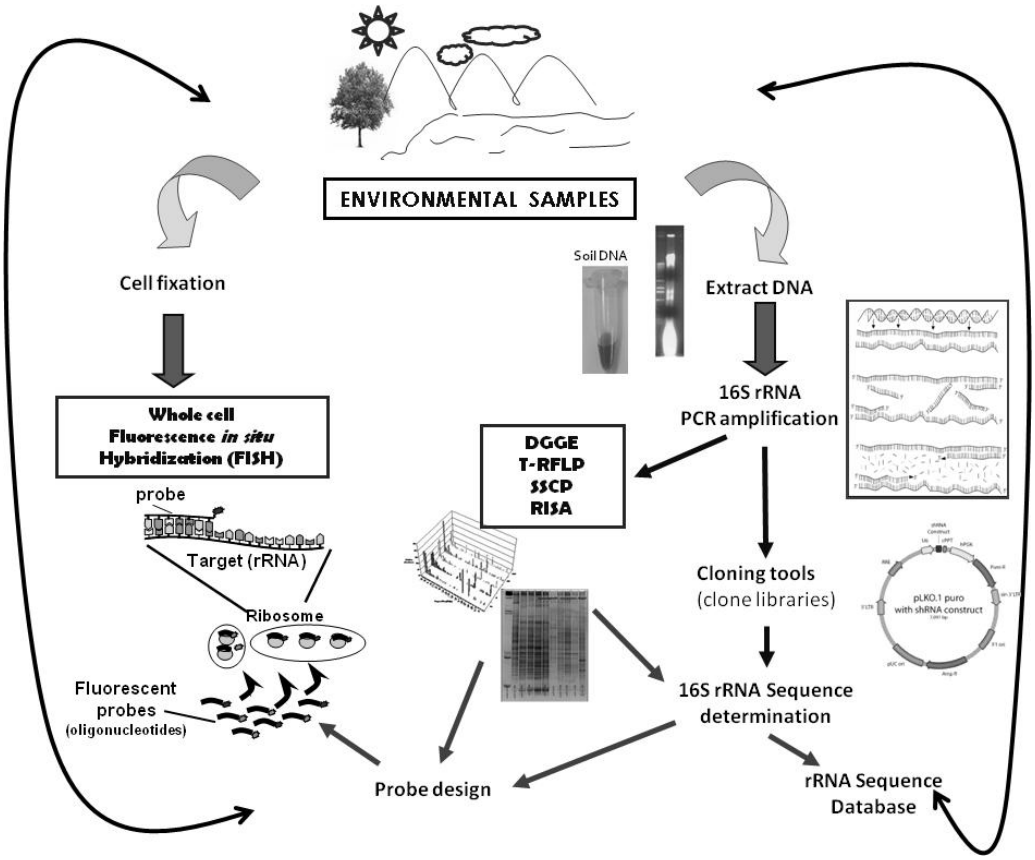


Figure 10. An overview of 16S rRNA based molecular methods for the analysis of soil microbial communities. PCR: Polymerase chain reaction; DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; SSCP: Single-strand conformation polymorphism; RISA: Ribosomal intergenic spacer analysis.

as denaturing gradient gel electrophoresis (DGGE) [60], terminal restriction fragment length polymorphism (T-RFLP) [61] or clone libraries [62] indicate the biodiversity and the structure of microbial communities in the environment. In addition, fluorescent *in situ* hybridization (FISH) [63, 64] uses fluorochrome-labeled oligonucleotides to identify *in situ* specific active microorganisms, obtaining direct measures of their relative abundance. These molecular methods have been used for the analyses of the microbial communities of pesticide-treated soils and the dynamics of microbial communities during bioremediation [65].

Soils are hot spots for microbial diversity [58, 66]. The most predominant bacterial phylogenetic groups in soil are *Proteobateria* (alpha-, beta- and gamma-subclasses), *Acidobacteria, Actinobacteria, Planctomycetes, Cytophagales*, and *Verrucomicrobia* [66]. In recent years, shifts in the bacterial community structure in soil promoted by pesticide application have been described [47, 67-69]. The bacterial community structure of a *p*-nitrophenol-contaminated site and the changes induced in the community structure during bioaugmentation with *Arthrobacter protophormiae* RKJ100 has been studied [65]. The microbial community of this pesticide-contaminated soil was mainly constituted by *Proteobacteria* and *Actinomycetes*. Although bioaugmentation with strain RKJ100 enhanced pollutant degradation, T-RFLP analysis revealed non-significant changes in bacterial community structure during the bioremediation process.

In *s*-triazine contaminated soils, the dynamics of microbial communities throughout the pesticide degradation processes has been described. In atrazine-treated agricultural soils, long term application of organic amendment (sewage sludge or waste water) changed the microbial community [70]. However, organic amendment did not modify atrazine-degrading activity of soils. The addition of atrazine and inorganic nitrogen source increased atrazine-degrading microorganism [71]. On the other hand, the effect of simazine and urea on the bacterial community structure in soils have been analysed by FISH [69]. Alpha- and Beta-*Proteobacteria* were the most influenced phylogenetic groups by the application of simazine and urea.

A recent report established that bioaugmentation with *Pseudomonas* sp. strain MHP41 promotes bacterial community changes in agricultural soils [47]. FISH analysis reported by Morgante *et al.* [47] revealed that bioaugmentation increased the relative abundances of two phylogenetic groups (*Acidobacteria* and *Planctomycetes*) in soils. The bacterial community response could be a consequence of the enhanced nitrogen bioavailability after simazine bioremediation [17, 25, 71, 72]. Microbial communities involved in herbicide degradation has to be further studied.

Research needs

In the last twenty five years, *s*-triazine-degrading bacteria have been isolated in diverse regions of the world. Recently, novel interesting strains have been isolated and an efficient *s*-triazine-degrading bacterium has been used for bioremediation studies. Native bacteria are potential biocatalysts for the bioremediation of polluted sites. Therefore, it will be useful to further isolate novel microbes able to degrade herbicides for recently polluted sites and for extreme environments that are contaminated with herbicides.

The *s*-triazine catabolic pathways in bacteria are still under evolution. The metabolic pathways in diverse bacteria have to be determined for the knowledge of the metabolic diversity and the key catabolic enzymes. The regulation of the metabolic pathways of *s*-triazines is not well known. In strain *Pseudomonas* sp. ADP it has been shown that only the *atzDEF* are clustered in an operon and are regulated by a transcriptional regulator and by nitrogen sources. The regulation of metabolic pathways for *s*-triazine degradation has to be further studied in other bacterial strains. Genomic studies of *s*-triazine-degrading bacteria will be of interest to unravel the metabolic pathways of *s*-triazines and its general regulation.

Microbial communities involved in the degradation of *s*-triazine in soils have to be further studied for the understanding of their role in this complex process and for the identification of the main microbial players in biodegradation of these compounds. Culture independent methods such as stable isotope monitoring as well as functional metagenomic approaches will be useful for these purposes. Effect of pesticides on other biogeochemical cycles in soil has to be determined. The cycles of diverse elements such as carbon, nitrogen, phosphorous and sulphur could be influenced by the pesticide application and by bioremediation processes.

Bioremediation technology for herbicides has to be further developed. Each polluted environment requires a specific bioremediation process. Novel biocatalysts and bioremediation processes are required for recently polluted sites, soils with different environmental properties and extreme environments contaminated with pesticides. Novel bioremediation processes will be employed to clean up pesticide polluted sites for a sustainable development.

Acknowledgements

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Appendix

Patent

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(19) **United States**

(12) **Patent Application Publication**

Hernandez et al.

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(43) **Pub. Date: Jan. 15, 2009**

(54) **S-TRIAZINE-HERBICIDE-DEGRADING BACTERIA, PRODUCT FOR THE BIOREMEDIATION AND METHOD OF BIOREMEDIATION**

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(52) **U.S. Cl.** **435/253.3**; 435/262.5

(57) **ABSTRACT**

The present invention provides a bacterial strain which is able to degrade or mineralize s-triazine compounds, such as simazine, which corresponds to *Pseudomonas* sp. strain MHP41, deposited under the accession number NRRL B-30908. The present invention provides a product for the bioremediation of environments contaminated with s-triazine, where the product includes a bacterial inoculum of *Pseudomonas* sp. strain MHP41. The present invention further provides a method for the bioremediation of environments contaminated with s-triazines, which uses this product for the bioremediation.

FIGURE 1

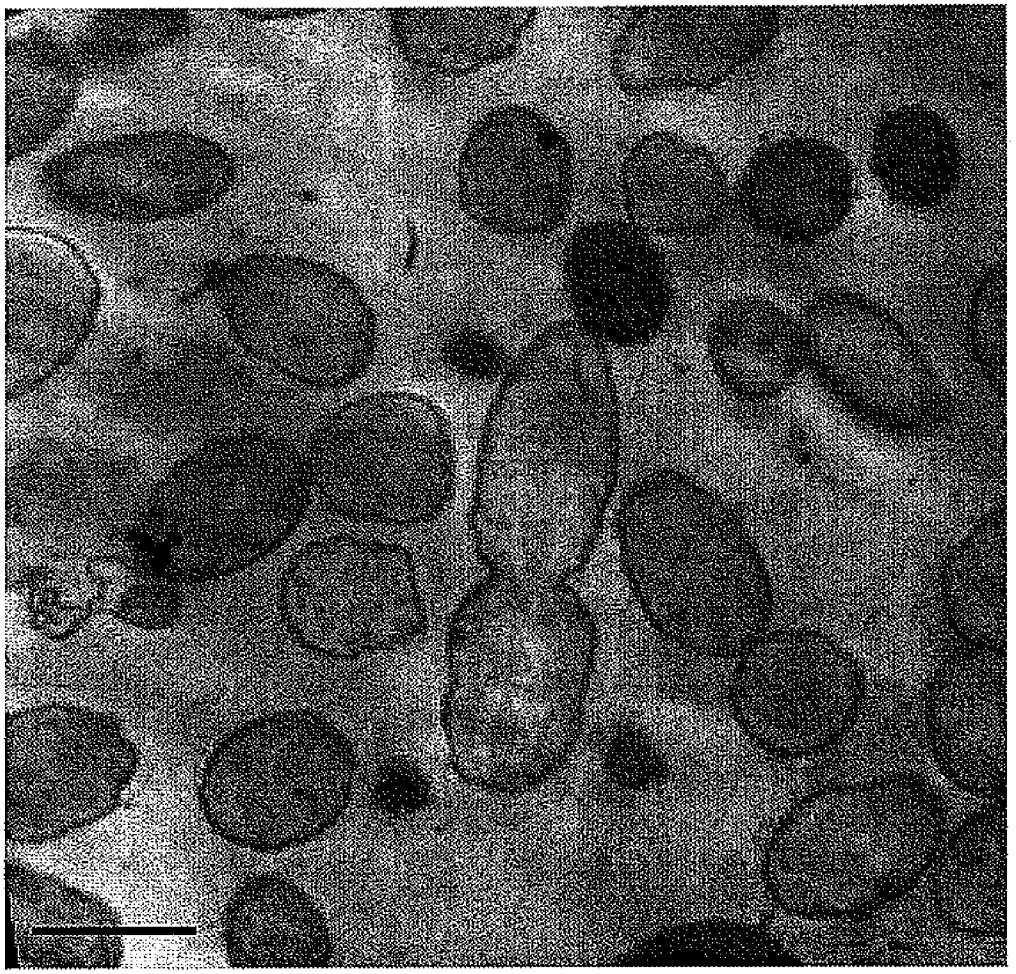


FIGURE 2

FIGURE 3

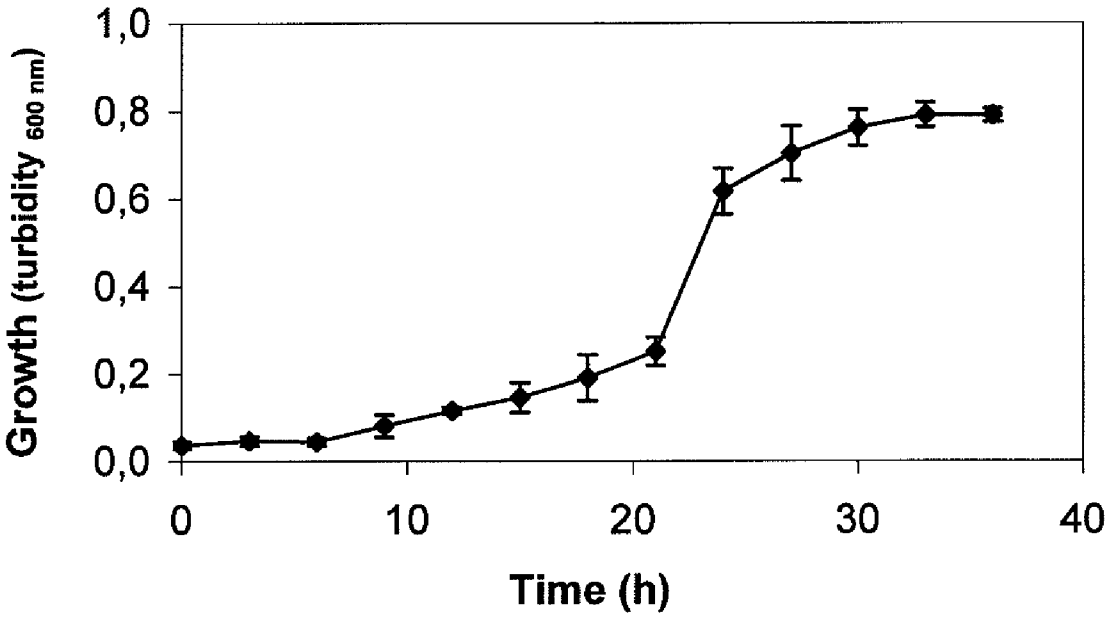
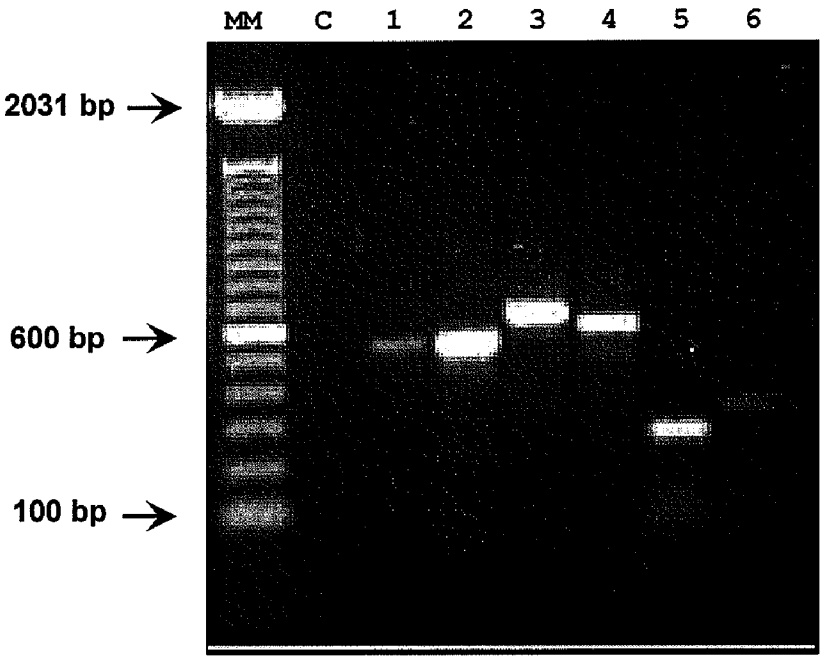
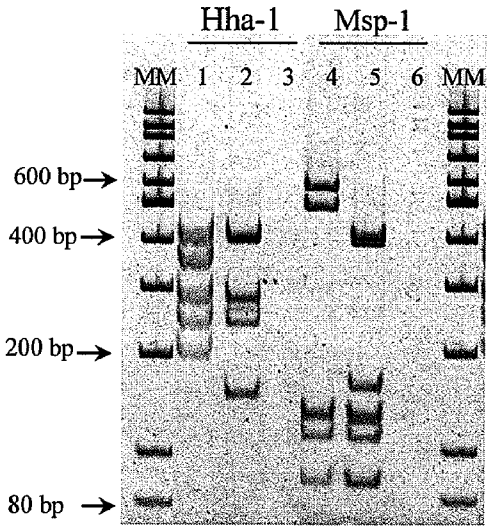


FIGURE 4



MM 100 bp
Negative control
1: *atzA*
2: *atzB*
3: *atzC*
4: *atzD*
5: *atzE*
6: *atzF*

FIGURE 5



MM: Molecular weight markers (100 bp)

Lanes **1 and 4**: *Pseudomonas* sp. ADP

Lanes **2 and 5**: *Pseudomonas* sp. MHP41

Lanes **3 and 6**: negative controls

FIGURE 6

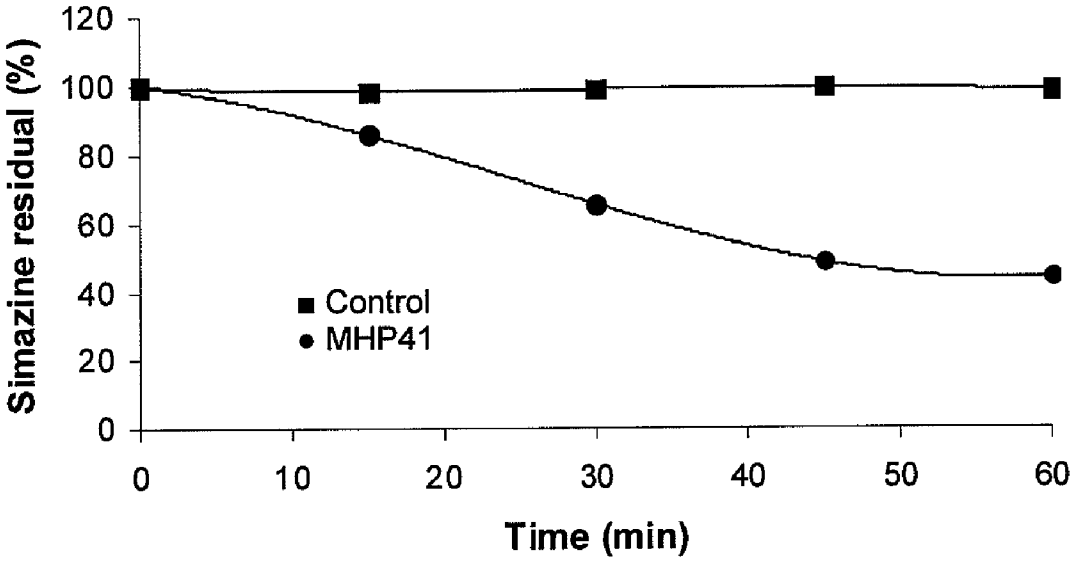

Simazine residual (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)
Control
MHP41

FIGURE 7

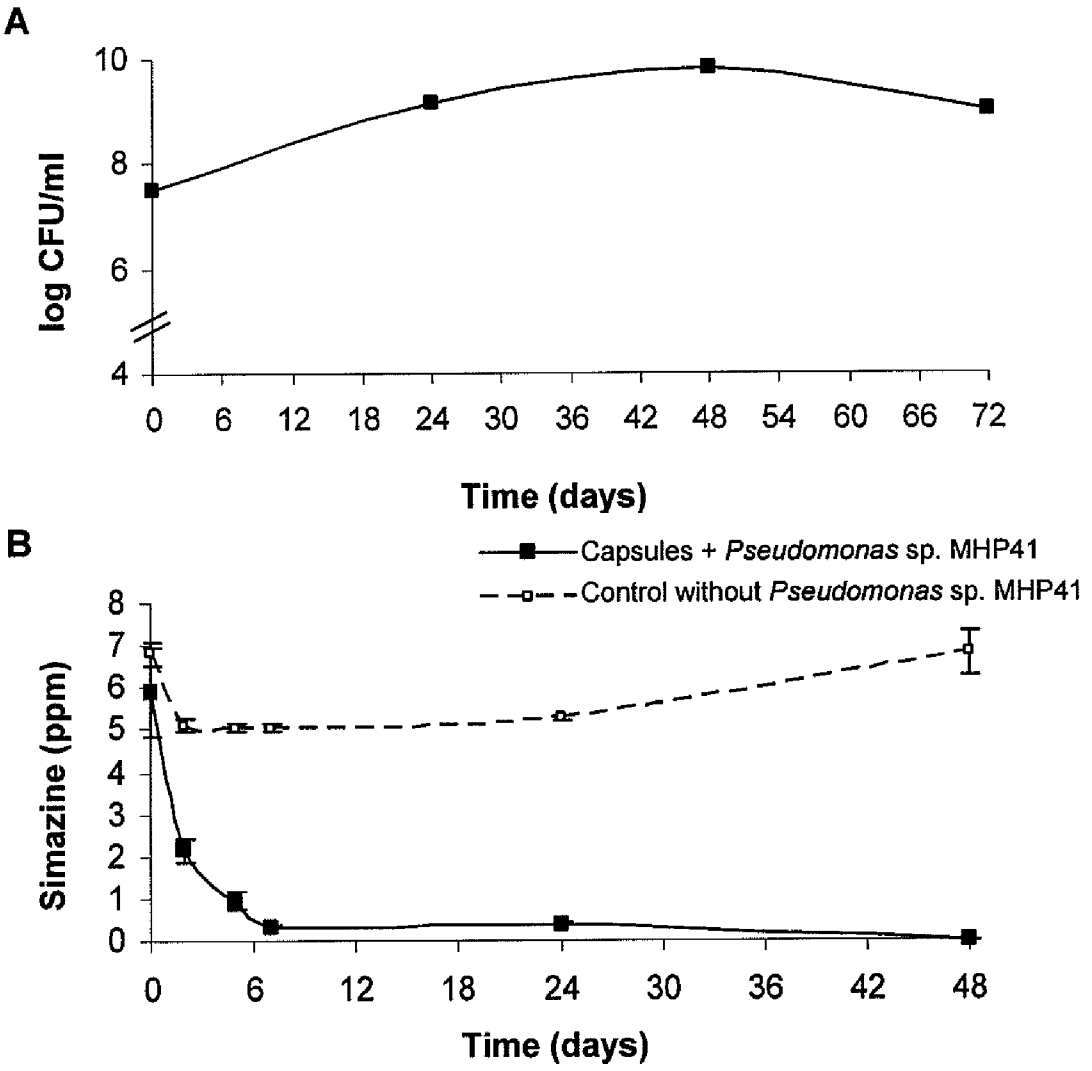

A
log CFU/ml
10
8
6
4
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (days)
B
Capsules + Pseudomonas sp. MHP41
Control without Pseudomonas sp. MHP41
Simazine (ppm)
8
7
6
5
4
3
2
1
0
0
6
12
18
24
30
36
42
48
Time (days)

FIGURE 8

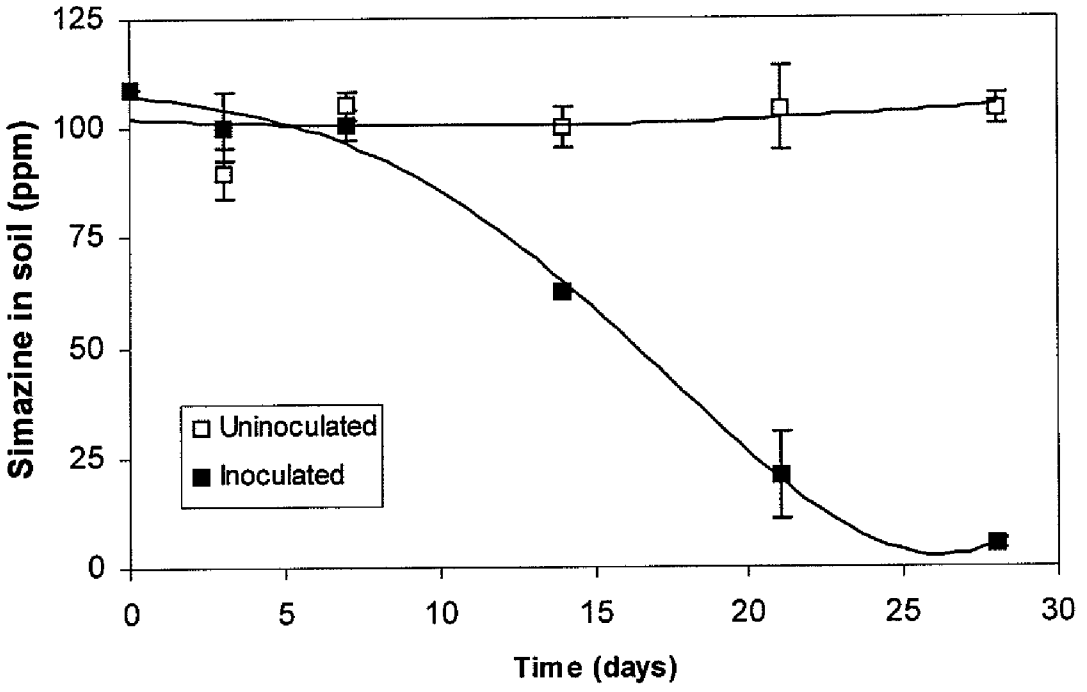
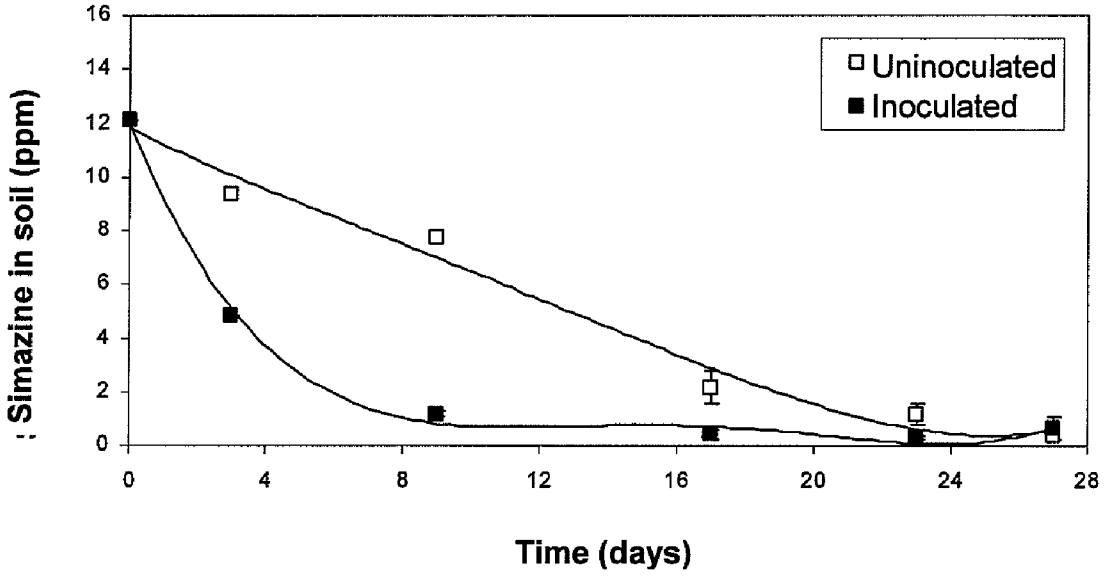

125
100
75
50
25
0
Simazine in soil (ppm)
Uninoculated
Inoculated
0
5
10
15
20
25
30
Time (days)

FIGURE 9


Uninoculated
Inoculated
Simazine in soil (ppm)
Time (days)
0
2
4
6
8
10
12
14
16
0
4
8
12
16
20
24
28

S-TRIAZINE-HERBICIDE-DEGRADING BACTERIA, PRODUCT FOR THE BIOREMEDIATION AND METHOD OF BIOREMEDIATION

[0001] The present invention relates to a bacterial strain that has the ability to degrade s-triazine herbicides, a product that contains this microorganism and an application method of said product for bioremediation of s-triazine-contaminated environments.

BACKGROUND OF THE INVENTION

[0002] s-Triazines are a family of herbicides that has a symmetric heterocyclic aromatic ring with 6 alternating carbon and nitrogen atoms. Simazine and atrazine are the most relevant members of the s-triazine herbicide family among other less-used compounds such as hydroxysimazine, deethylhydroxysimazine, hydroxyatrazine, deethylatrazine, deethylhydroxyatrazine, deisopropylatrazine, fluoroatrazine, propazine, terbutylazine, cyanuric acid and cyanazine. Both simazine (IUPAC: 6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine) and atrazine (IUPAC: 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) are s-triazine herbicides that have a chlorine atom bound to the heterocyclic ring. The mechanism of this herbicide family comprises the inhibition of electron transport in the photosynthesis process.

[0003] s-Triazines, especially simazine and atrazine, have been widely used for the control of weeds in many agricultural regions of the world. In Chile, simazine is commonly used for agricultural and forestry practices.

[0004] In spite of their advantageous use as herbicides, s-triazines are environmental contaminants that can affect human health and ecosystems. It has been shown that atrazine and other s-triazines can induce breast cancer in rats. Therefore they have been classified as "possible human carcinogen" class-C by EPA (Tappe et al., 2002). Various ecosystems have been chemically perturbed by the constant use of s-triazine herbicides, especially simazine and atrazine. Due to their high mobility, these compounds can cause serious soil and ground water contamination. Therefore these herbicides frequently exceed the standard advisory levels of 0.1 $\mu g\ L^{-1}$ and 3.0 $\mu g\ L^{-1}$ by the European Union and USA regulatory authorities, respectively (Rousseaux et al., 2001).

[0005] Despite this background, the global manufacture and sales of herbicides have raised and showed an increase trend from the 1980 decade. Statistics of the United Nations Food and Agriculture Organization carried out from the early XXI century reveal that herbicide consumption, and especially s-triazines, is high both in the European Union and in the US. According to these statistics, US, France and Germany are the main countries that export s-triazine herbicides.

[0006] According to the Environmental Protection Agency (EPA), the global market for pesticides during years 2000 and 2001 was more than 2.3 million tons. Only in the US 0.7 million tons were consumed during years 2000 and 2001, which represents about 40% of the herbicide world market and more than 30% of the pesticide world market. In the US, atrazine is the most employed herbicide for weed control in corn, citric, vine and fruit tree plantations, whereas simazine is mainly used in corn crops.

[0007] Due to the extensive use of s-triazines for weed control, simazine and atrazine presence has been detected in surface and ground water in several countries such as the US, Switzerland, South Africa and Germany. In Chile, according to the Servicio Agrícola Ganadero de Chile SAG) (Declaración de ventas de plaguicidas de uso agrícola, Ministerio de Agricultura de Chile, 2006), the pesticide consumption during 2004 was about 23,000 tons. Important agricultural plantations (avocado, citruses and grape) in Chile are treated with pre-emergent herbicides such as atrazine or simazine. The use of these herbicides reached 350 tons, representing about 10% of the herbicide used in the country. In the last time, the massive use of agrochemicals has motivated the study of environments that are potentially affected by these compounds. Recent studies have detected s-triazine compounds in agricultural watershed at south-central Chile.

[0008] Therefore environmental contamination by s-triazines is a worldwide problem, which requires urgent attention.

[0009] One interesting alternative to mitigate s-triazines contamination is bioremediation, which employs microorganisms able to degrade these pollutants or transform them into innocuous compounds for the environment and human health. Some s-triazine-degrading consortia or pure bacterial strains have been isolated, such as *Pseudaminobacter, Nocardioides* sp. and *Agrobacterium. Pseudomonas* sp. ADP strain (Mandelbaum and Wacket, 1996) is able to mineralize s-triazines due to specific catabolic pathways well described in the state of the art. Probably, this is the best-known and one of the most characterized strains of the atrazine-degrading microorganisms.

[0010] Nevertheless, efficient microorganisms able to degrade s-triazines in the environment are still required.

[0011] This technical problem has been solved in the present invention, by the selection of a native bacterium able to degrade s-triazines, i.e. *Pseudomonas* sp. strain MHP41. In addition, the present invention describes a product that contains strain MHP41 and a method for the bioremediation of s-triazine-polluted environments that uses said product.

SUMMARY OF THE INVENTION

[0012] The present invention relates to a bacterial strain, which is capable to degrade s-triazine herbicides.

[0013] In one aspect of the present invention, these s-triazine compounds are selected from the group consisting of chlorinated compounds, wherein simazine and atrazine were selected from the chlorinated s-triazines.

[0014] In a preferred embodiment of the present invention, the bacterial strain *Pseudomonas* sp. strain MHP41 which has been deposited in a microorganism collection called Agricultural Research Culture Collection (NRRL) from Peoria, Ill., United States, under the access number B-30908, on Mar. 17, 2006, which is able to grow and completely degrade simazine.

[0015] Furthermore, the present invention discloses a product containing this microorganism.

[0016] The present invention also comprises a method for the treatment or bioremediation of an environment contaminated with s-triazine-compounds wherein this method comprises the stages of: i) adding *Pseudomonas* sp. strain MHP41 (NRRL B-30908) to said s-triazine-polluted environment, where this bacterium is able to degrade s-triazine compounds, and ii) incubating this bacterium, *Pseudomonas* sp. strain MHP41 (NRRL B-30908), in the contaminated environment during a period of time sufficient to permit the complete degradation the s-triazine compounds present in the environment and the bioremediation thereof.

[0017] The present invention also discloses *Pseudomonas* sp. strain MHP41 (NRRL B-30908), a native bacterial strain isolated from soil. This bacterium belong to the genus *Pseudomonas*, is easily cultivated, reaches high cellular densities and shows fast grow under laboratory conditions. This strain grows at 30° C. from a turbidity measured at 600 nm of 0.082 (1×10^5 CFU/ml) to a turbidity at 600 nm of 1.012 (2×10^8 CFU/ml), in minimal medium using simazine as the single nitrogen source.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. **1** shows a micrograph of *Pseudomonas* sp. strain MPH41. The micrograph was obtained by transmission electronic microscopy. The bar represents 1 μm.

[0019] FIG. **2** shows a photograph of a colonies of *Pseudomonas* sp. strain MPH41 growing on minimal medium agar plates with simazine as the sole nitrogen source. Simazine degradation is visualized by clearing zones around the colonies.

[0020] FIG. **3** shows the growth of *Pseudomonas* sp. strain MPH41 using simazine as the sole nitrogen source. The growth was followed measuring turbidity at 600 nm and the growth values are the mean of 3 independent experiments.

[0021] FIG. **4** shows the detection of atrazine catabolic atz genes in *Pseudomonas* sp. strain MHP41 by PCR using specific primers. PCR-amplification products: atzA (lane 1), atzB (lane 2), atzC (lane 3), atzD (lane 4), atzE (lane 5) and atzF (lane 6) of *Pseudomonas* sp. strain MHP41.

[0022] FIG. **5** shows the restriction profile of the ribosomal 16S rRNA gene of strains *Pseudomonas* sp. MHP41 and *Pseudomonas* sp. ADP. In this figure, lanes 2 and 5 correspond to *Pseudomonas* sp. MHP41, lanes 1 to 4 correspond to *Pseudomonas* sp. ADP and lanes 3 and 6 correspond to negative controls. 16S rRNA gene PCR-amplification products were digested with HhaI and MspI restriction enzymes. 100 pb molecular mass (MM) marker, is shown at the left side.

[0023] FIG. **6** shows the simazine-degradation kinetics by *Pseudomonas* sp. strain MHP41. The values indicate the mean of 3 independent experiments.

[0024] FIG. **7** shows the growth and simazine degradation by immobilized *Pseudomonas* sp. MHP41 in sodium alginate. A. Growth (turbidity at 600 nm) of strain MHP41 immobilized in sodium alginate matrix. B. Simazine degradation in aqueous medium. Microcapsules were incubated in AM minimal medium with simazine as the sole nitrogen source. Each value is the mean of three independent experiments.

[0025] FIG. **8** shows simazine degradation kinetics by *Pseudomonas* sp. strain MHP41 during bioremediation in soil microcosms without history of simazine application. During bioaugmentation with encapsulated *Pseudomonas* sp. strain MHP41, 50% of the herbicide is degraded after 15 days. Herbicide attenuation is not observed in control soil after 28 days.

[0026] FIG. **9** shows simazine degradation kinetic by *Pseudomonas* sp. strain MHP41 during bioremediation in soil microcosms with history of simazine application. Three days after *Pseudomonas* sp. strain MHP41 application, 50% of the applied simazine was attenuated. By bioaugmentation, 90% of the applied simazine is removed in soil after 15 days. In non-inoculated soil, simazine removal takes at least 25 days.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

[0027] The present invention is related to a bacterial strain which is able to degrade s-triazine compounds, a product that contains this strain and a bioremediation method for environments polluted with s-triazine compounds.

DEFINITIONS

[0028] As used in the present invention the term "s-triazines" refers to molecules having a basic structure that comprises a symmetric heterocyclic aromatic ring with 6 alternating carbon and nitrogen atoms.

[0029] As used in the present invention the term "simazine" refers to a s-triazine molecule having carbon atoms substituted with one chlorine atom and two ethylamine groups (IUPAC: 6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine).

[0030] As used in the present invention the term "atrazine" refers to a s-triazine molecule having carbon atoms substituted with one chlorine atom, an ethylamine group and an isopropylamine group (IUPAC: 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine).

[0031] As used in the present invention the term "degrade" represents the decomposition of a chemical compound, such as s-triazine compound, by a metabolic pathway, to obtain a molecule with lower complexity.

[0032] As used in the present invention the term "mineralize" means biological decomposition of an organic compound, such as s-triazine compound, into molecules of minimal complexity.

[0033] As used in the present invention the term "bioremediation" means a treatment method to treat an environment or material considered as contaminated waste material located in a defined environment, where said treatment allows transforming this waste material into a less toxic material for the surrounding environment, or transforming this waste material into a material which can be metabolized by a microorganism or group of microorganisms, where this treatment method comprises the application of a living microorganism as a component of the treatment method.

[0034] As used in the present invention the term "native bacterium" means a natural bacterium isolated from soil and that were not modified genetically.

[0035] As used in the present invention, the term "inoculum" means a concentrated bacterial suspension (free or encapsulated cells) of a known concentration (CFU/ml).

[0036] As used in the present invention the term "ribotype" means an identity classification and species and subspecies differentiation based on the RFLP (Restriction Fragment Length Polymorphism) analysis of the 16S rRNA gene.

[0037] As used in the present invention the term "buffer solution" means a solution which is able to keep the pH value when acid or base are added.

[0038] As used in the present invention the term "lyophilized" refers to a *Pseudomonas* sp. strain MHP41 inoculum subjected to a lyophilization process, which is a method that consists of a fast dehydration under vacuum and at low temperatures, to achieve better product conservation.

[0039] As used in the present invention the term "microcosms" refers to a certain volume of soil in a container, whose important variables such as moisture, temperature and presence or absence of microorganisms and/or organic contaminants are known and controlled.

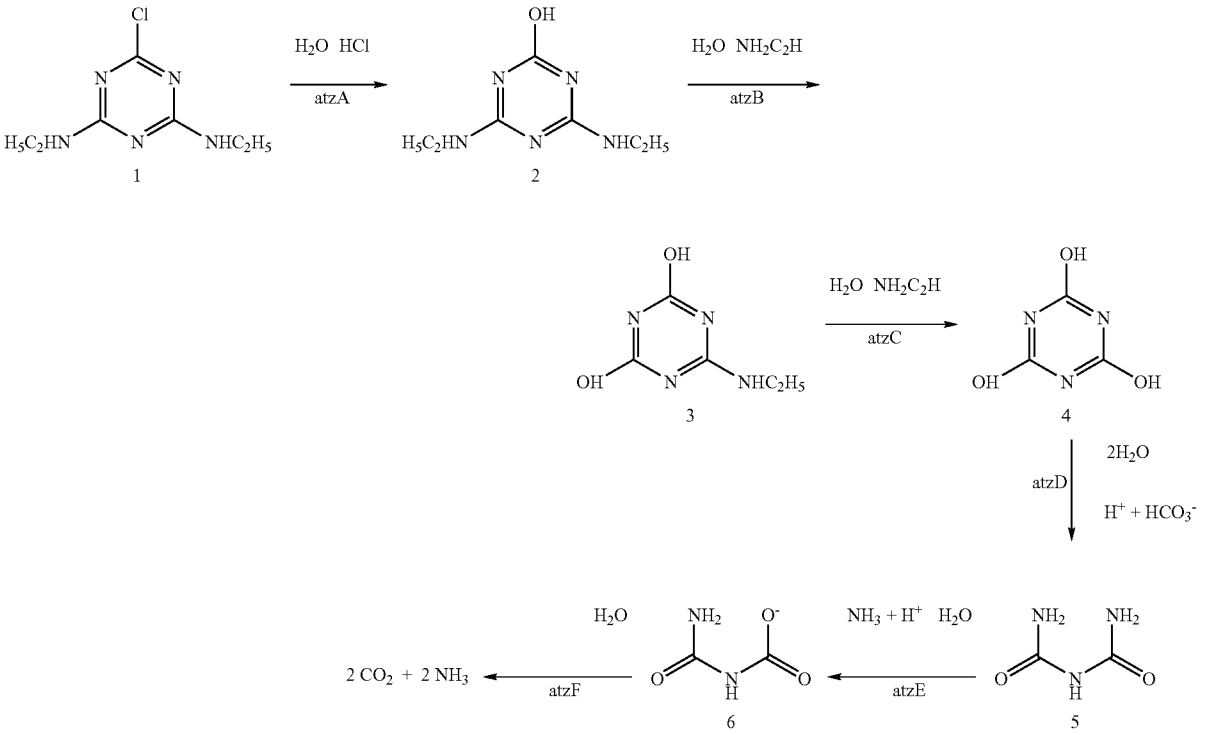
[0040] To obtain a bacterial strain able to degrade s-triazine compounds, microorganisms were isolated from agricultural soil samples and selected in a culture medium with simazine as the only nitrogen source. Bacteria were isolated in minimal

agar plates with simazine as the only nitrogen source, and individual colonies were isolated from the plates.

[0041] A native strain was isolated which is able to degrade s-triazine compounds and to use simazine as the only nitrogen source. This strain was designated as MHP41. The bacterium was identified as a *Pseudomonas* sp., based on a sequence analysis of the 16S rRNA gene and named as *Pseudomonas* sp. strain MHP41.

[0042] As was previously discussed in the background of the invention, diverse microorganisms that are able to degrade s-triazines are known in the state of the art, such as *Pseudomonas* sp. ADP. In this microorganism, the s-triazine degradation pathway has been widely studied and characterized, and their catabolic genes are well known. It is probable that another strain able to degrade s-triazines uses the same metabolic pathway and genes. Diagram 1 represents the aerobic process of s-triazine degradation.

Diagram 1.



way involves the enzymes cyanuric acid amidohydrolase (AtzD), biuret hydrolase (AtzE) and allophanate hydrolase (AtzF), which are encoded by the atzD, atzE and atzF genes, respectively. These enzymes transform the compound cyanuric acid to biuret (5) and allophanate (6) and finally into carbon dioxide and ammonia (de Souza et al., 1998; Cheng et al., 2005).

[0044] *Pseudomonas* sp. strain MHP41 possesses the atzA, atzB, atzC, atzD, atzE and atzF genes. Therefore, the s-triazine degradation pathways of strain MHP41 are similar to the pathways that have been described in the state of the art.

Description of *Pseudomonas* sp. Strain MHP41.

[0045] The new *Pseudomonas* sp. strain MHP41 is a Gram-negative, motile, rod-shaped bacterium. It was catalase positive and oxidase negative. The strain MHP41 is able to grow on plates with simazine or atrazine as nitrogen source, showing clearing zones around the colonies that indicate simazine degradation.

[0046] The present strain was identified by using the Biolog identification test and the analysis of the 16S rRNA gene sequence. MHP41 was identified as a strain belonging to the genus *Pseudomonas*.

[0047] This strain is sensitive to the antibiotics rifampicin, kanamycin, carbamicillin, tetracycline, and streptomycin, and has natural resistance to ampicillin.

[0048] The metabolic profile of *Pseudomonas* sp. strain MHP41 is presented in Table 1.

TABLE 1

Carbon sources for metabolism (respiration) of *Pseudomonas* sp. strain MHP41. Studies based on the Biolog microplates identification system.

Carbon sources on which *Pseudomonas* sp. MHP41 metabolize	Dextrin		L-Proline
	Glycogen	Propionic Acid	D-Serine
	Tween 40	Shikimic Acid	L-Serine
	Tween 80	Sebacic Acid	L-Threonine
	N-acetyl-D-glucosamine	Succinic Acid	D,L-Carnitine
	α-D-Glucose	Methyl Pyruvate	γ-Aminobutyric Acid
	Acetic Acid	Bromosuccinic Acid	Urocanic Acid
	Cis-Aconitic Acid	L-Alaninamide	Phenylethylamine
	Citric Acid	D-Alanine	Putrescine
	Formic Acid	L-Alanine	2-Aminoethanol
	D-Gluconic Acid	L-Alanilglycine	Monomethyl Succinate
	α-Hydroxybutyric acid	L-Asparagine	L-Leucine
	β-Hydroxybutyric Acid	L-Aspartic Acid	L-Ornithine
	Itaconic Acid	L-Glutamic Acid	α-Ketoglutaric Acid
	α-Ketobutyric Acid	Glycyl-L-glutamic Acid	α-Ketovaleric Acid
	D,L-Lactic Acid	Hydroxy-L-Proline	
Carbon sources on which *Pseudomonas* sp. MHP41 does not metabolize	α-Cyclodextrin	D-Melibiose	D-Saccharic Acid
	N-Acetyl-D-galactosamine	β-Methyl D-Glucoside	Succinamic Acid
	Adonitol	D-Psicose	Glucuronamide
	L-Arabinose	D-Raffinose	Glycyl-L-aspartic Acid
	D-Arabitol	L-Rhamnose	L-Histidine
	D-Cellobiose	D-Sorbitol	L-Phenylalanine
	i-Erithritol	Sucrose	L-Pyroglutamic Acid
	L-Fructose	D-Trehalose	Inosine
	L-Fucose	Turanose	Uridine
	D-Galactose	Xylitol	Thymidine
	Gentiobiose	Malonic Acid	2,3-Butanediol
	m-Inositol	D-Galactonic Acid Lactone	Glycerol
	α-D-Lactose	D-Galacturonic Acid	D,L-α-Glycerol Phosphate
	Lactulose	D-Glucosaminic Acid	Glucose-1-phosphate
	Maltose	D-Glucuronic Acid	Glucose-6-phosphate
	D-Mannitol	γ-Hydroxybutyric Acid	D-Mannose
	p-Hydroxyphenylacetic Acid		

[0049] *Pseudomonas* sp. strain MHP41 possesses the atzA, atzB, atzC, atzD, atzE and atzF genes for s-triazine degradation which confer the capability to degrade diverse s-triazine compounds (Table 2). The AtzA enzyme catalyzes the hydrolytic removal of chlorinated and fluorinated substituents from s-triazine molecules, but is unable to remove cyano, azide, methoxy, thiomethyl or amino groups from compounds that are structurally similar to simazine or atrazine.

TABLE 2

Table 2. Chemical structures of s-triazine compounds degraded by the native strain *Pseudomonas* sp. MHP41.

Substituents				
R1	R2	R3	s-triazines	Degradation
Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	Simazine	+
OH	$NHCH_2CH_3$	$NHCH_2CH_3$	Hydroxysimazine	+
OH	NH_2	$NHCH_2CH_3$	Deethylhydroxysimazine	+
Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	Atrazine	+

TABLE 2-continued

Table 2. Chemical structures of s-triazine compounds degraded by the native strain *Pseudomonas* sp. MHP41.

Substituents				
R1	R2	R3	s-triazines	Degradation
OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	Hydroxyatrazine	+
Cl	NH_2	$NHCH(CH_3)_2$	Deethylatrazine	+
OH	NH_2	$NHCH(CH_3)_2$	Deethylhydroxyatrazine	+
Cl	$NHCH_2CH_3$	NH_2	Deisopropylatrazine	+
Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	Propazine	+
Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$	Terbutylazine	+
OH	OH	OH	Cyanuric acid	+
Cl	$NHCH_2CH_3$	$NHC(CH_3)_2CN$	Cyanazine	+
F	$NHCH_2CH_3$	$NHCH(CH_3)_2$	Fluoroatrazine	+

[0050] FIG. **1** shows a micrograph of *Pseudomonas* sp. strain MPH41, the bacterium of the present invention. The micrograph was obtained by transmission electronic microscopy.

[0051] FIG. **2** shows a colony photograph of *Pseudomonas* sp. strain MPH41 growing on minimal medium agar plate with simazine as the sole nitrogen source. Simazine degradation is visualized by clearing zones around the colonies.

[0052] FIG. **3** shows the growth curve of *Pseudomonas* sp. Strain MPH41. Cells were grown in AM minimal medium using simazine as the sole nitrogen source and succinate as the sole carbon source. Growth was monitored by measuring turbidity at 600 nm with a Lambda 11 spectrophotometer (Perkin Elmer). Results were obtained for 3 independent experiments. Growth was observed for the native *Pseudomonas* sp. strain MHP41, reaching the exponential phase after 24 hours and reaching the stationary phase with a turbidity of 0.8 (1.5×10^5 CFU/ml) after 36 hours (FIG. **3**).

[0053] FIG. **4** shows the detection of the six degradation atz genes for s-triazines in *Pseudomonas* sp. strain MHP41. The amplification of the atz genes was carried out by PCR using specific primers previously described (de Souza et al., 1998). PCR products were analyzed by electrophoresis in agarose gel (1.2%) in TBE (0.5×) and were stained with ethidium bromide (FIG. **4**).

[0054] It is important to mention that the isolated *Pseudomonas* sp. strain MHP41, is different to the strain described in the state of the art, *Pseudomonas* sp. strain ADP. *Pseudomonas* sp. strain MHP41 has important genetic and phenotypic differences with the reference strain *Pseudomonas* sp. ADP (Mandelbaum et al., 1996). Strain MHP41 has a different 16S rRNA gene sequence that strain ADP. This is the most used gene for bacterial taxonomy. Strain MHP41 can be genetically modified in the lab, incorporating plasmids of incompatibility series IncP, IncQ and Cryptic with high frequency. To date, there are no studies that describe the genetic modification of *Pseudomonas* sp. strain ADP.

[0055] FIG. **5** shows the restriction profile of the 16S rRNA gene of the native strain *Pseudomonas* sp. MHP41 and the reference strain *Pseudomonas* sp. ADP. The Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA genes were done by digestion with the restriction enzymes Msp-1 and Hha-1. Digestion with both enzymes generates differences in the restriction profiles of the 16S rRNA genes from both strains. Therefore, *Pseudomonas* sp. MHP41 and *Pseudomonas* sp. ADP are different ribotypes (FIG. **5**).

[0056] In an additional embodiment, the present invention provide a product and a treatment method to improve the bioremediation of a contaminated environment with s-triazine herbicides such as simazine, hydroxysimazine, deethylhydroxysimazine, atrazine, hydroxyatrazine, deethylatrazine, deethylhydroxyatrazine, deisopropylatrazine, fluoroatrazine, propazine, terbutylazine, cyanuric acid and cyanazine.

[0057] In addition, the product provided by the present invention comprises an inoculum of the native *Pseudomonas* sp. strain MHP41 and a culture medium or a buffer solution or sodium alginate. This inoculum contains a known concentration of *Pseudomonas* sp. strain MHP41 (NRRL B-30908) ranging from 1×10^3 CFU/ml to 1×10^{15} CFU/ml of culture medium or buffer solution, or from 1×10^5 CFU/g to 1×10^{12} CFU/g of sodium alginate. Preferentially, the bacteria of the present invention have been previously grown in a minimal medium with simazine as the only nitrogen source.

[0058] In a preferred embodiment, the product provided by the present invention comprises an inoculum of *Pseudomonas* sp. strain MHP41 and the bacterial cells are encapsulated in a sodium alginate matrix. This immobilization protects bacterial cells from adverse environmental conditions, decreases their exposure to toxic compounds, and thus increases their stability and viability (Cassidy et al., 1996). This product for s-triazine bioremediation contains an inoculum of the bacteria *Pseudomonas* sp. strain MHP41 at a concentration which ranges from about $\times10^5$ CFU/g to about 1×10^{12} CFU/g of alginate.

[0059] In an additional embodiment of the present invention, the product contains an inoculum of the lyophilized cells of native bacteria *Pseudomonas* sp. strain MHP41, which further improves their transport and commercialization. To obtain this product for bioremediation, the bacteria have been preserved in a latent state by lyophilization of a concentrated bacterial suspension using protective sterile complex media such as bovine serum, powdered defatted milk or yeast extract, an amino acid such as monosodium glutamate, a fatty acid such as meso-inositol, and also a carbohydrate such as glucose, lactose, raffinose or mannitol. The lyophilized inoculum can be directly incubated in a liquid or solid growth medium, preferably at 28° C. during 48 h to recover active cells. After this activation process, the previously lyophilized *Pseudomonas* sp. MHP41 cells are able to degrade s-triazines. The product for bioremediation contains this lyophilized inoculum of the bacteria which after suspension in a suitable volume yields a concentration ranging from about 10^5 CFU/ml to 10^{12} CFU/ml of medium.

[0060] The product of the present invention can also be formulated as a suspension of *Pseudomonas* sp. strain MHP41 in a liquid culture medium. Many culture media could be used for this purpose, and any of them could be used within the scope of the present invention, especially preferring minimal media described in Table 3, included in Example 2 of the present document. Said suspension comprises a known concentration of *Pseudomonas* sp. strain MHP41 NRRL B-30908 ranging from about 1×10^3 CFU/ml to 1×10^{15} CFU/ml, preferably from 1×10^5 CFU/ml to 1×10^{12} CFU/ml.

[0061] In a preferred embodiment of the invention, the bioremediation method comprises the addition of the product containing *Pseudomonas* sp. strain MHP41 to the environment contaminated with s-triazine compounds, such as simazine. In the method of the present invention, the bacteria are able to degrade completely the s-triazine compound, such as simazine. The product used for bioremediation can be applied as an inoculum of *Pseudomonas* sp. strain MHP41 encapsulated in a sodium alginate matrix, or an active suspension of the lyophilized product of *Pseudomonas* sp. strain MHP41 cells, or a suspension of *Pseudomonas* sp. strain MHP41 in a culture medium. After the inoculation into the contaminated environment of the product previously described in the embodiments of the present invention, the bacteria degrade the s-triazine compound of the contaminated environment during a period of time, from at least 1 week to about 12 months. Normally, a period from 1 to 4 weeks should be enough for an effective degradation of the contaminant. The concentration of *Pseudomonas* sp. strain MHP41 NRRL B-30908 in the contaminated environment which is usually soil, ranges from 1×10^2 CFU/g of soil to about 1×10^{12} CFU/g of soil.

Example 1

Isolation of *Pseudomonas* sp. Strain MHP41

[0062] The isolation of *Pseudomonas* sp. strain MHP41 was carried out by enrichment using simazine as the sole

nitrogen source. For the isolation AM minimal medium (Table 3) was used, which has a saline fraction providing the micronutrients, succinate as the sole carbon source and saturated simazine as the sole nitrogen source. For the initial enrichment culture, samples were taken directly from agricultural soils. Cultures were incubated at 28° C. and then subcultures were grown. From these subcultures, bacteria were isolated in minimal agar plates using simazine as the sole nitrogen source. Individual colonies were isolated from the agar plates. These strains were conserved in glycerol (20%) at −24° C.

nate was developed for the strain. For the lyophilization process, a protective sterile complex medium such as bovine serum, powdered defatted milk or yeast extract, an amino acid such as monosodium glutamate, a fatty acid such as meso-inositol, a carbohydrate such as glucose, lactose, raffinose or mannitol and also glycerol or dimethylsulfoxide are used.

[0066] In all the culture media and conservation methods, the viability and biodegrading capability of *Pseudomonas* sp. strain MHP41 is not reduced.

TABLE 3

Table 3. Minimal media formulations for maintenance of the native *Pseudomonas* sp. strain MHP41. To prepare solid media it is supplemented with 15 g/L agar.

Name	Composition (g/L)	Carbon and Nitrogen Sources
AM	K_2HPO_4 1.6 g; KH_2PO_4 0.4 g; $MgSO_4 \times 7H_2O$ 0.2 g; NaCl 0.1 g; $CaCl_2$ 0.02 g; 20 ml of trace element solution containing (per liter): EDTA 2.5 g; $ZnSO_4$ 11.1 g; $FeSO_4$ 5 g; $MnSO_4 \times H_2O$ 1.54 g; $CuSO_4 \times 5H_2O$ 0.4 g; $Co(NO_3)_2 \times 6H_2O$ 0.25 g; $Na_2B_4O_7 \times 10H_2O$ 0.18 g and H_2SO_4 5 ml.	Carbon Source: Sodium succinate 2.5 mM
Minimal medium (MM)	100 ml phosphate buffer 10×, 2 ml of microelement solution 500x. Phosphate buffer 1× contains (per liter): 70 g $Na_2HPO_4 \times 2H_2O$, 28 g KH_2PO_4 and 5 g NaCl. Microelement solution 500x contains (per 100 ml): 5 g $MgSO_4 \times 7H_2O$, 0.5 g $FeSO_4 \times 7H_2O$, 0.25 g $MnSO_4 \times H_2O$, 0.32 g $ZnCl_2$, 0.033 g $CaCl_2 \times 2H_2O$, 0.018 g $CuSO_4 \times 5H_2O$, 0.015 g $CoCl_2 \times 6 H_2O$, 0.325 g H_3BO_3, 0.5 g EDTA, 7.3 ml HCl 37%.	Nitrogen Source: Simazine 0.5 mM For solid media 3 mM final concentration was used.
BSMA	K_2HPO_4 0.5 g; $MgSO_4 \times 7H_2O$ 0.5 g; $FeCl_3 \times H_2O$ 10 mg; $CaCl_2 \times H_2O$ 10 mg; $MnCl_2$ 0.1 mg; $ZnSO_4$ 0.01 mg.	
Brunner	Na_2HPO_4 2.44 g; KH_2PO_4 1.52 g; $MgSO_4 \times 7H_2O$ 0.2 g; $CaCl_2 \times 2H_2O$ 0.05 g; 10 ml o fan element trace solution containing (per liter): EDTA 0.5 g; $FeSO_4 \times 7H_2O$ 0.2 g; 100 ml of an element trace solution containing (per liter): $ZnSO_4 \times 7H_2O$ 0.1 g; $MnCl_2 \times 4H_2O$ 0.03 g; H_3BO_3 0.3 g; $CoCl_2 \times 6H_2O$ 0.2 g; $CuCl_2 \times 2H_2O$ 0.01 g; $NiCl_2 \times 6H_2O$ 0.02 g; $Na_2MoO_4 \times 2H_2O$ 0.03 g.	

[0063] After this procedure, the native strain described in the present invention was obtained, i.e. *Pseudomonas* sp. MHP41. This bacterial strain has the capability to grow using simazine as the sole only nitrogen source. This strain was deposited in the microorganism collection Agricultural Research Culture Collection (NRRL) at Peoria, Ill., United States, under the accession number NRRL B-30908, on Mar. 17, 2006.

Example 2

Maintenance and Conservation of the Product Containing *Pseudomonas* sp. MHP41

[0064] *Pseudomonas* sp. strain MHP41 is kept viable in the laboratory by culturing in different minimal media which composition is detailed in Table 3.

[0065] As alternative conservation methods, lyophilization processes and an encapsulation processes using sodium algi-

Example 3

Degradation of the Simazine Compound by *Pseudomonas* sp. MHP41

[0067] The ability of *Pseudomonas* sp. MHP41 to degrade simazine was assessed. Therefore, the bacterial strain MHP41 was cultured in AM medium using simazine as the sole nitrogen source at 30° C. for 16-20 h until the exponential phase (turbidity at 600 nm of 0.3; 7×10^7 CFU/ml). Cells were centrifuged and washed with sodium phosphate buffer [60 mM sodium phosphate (pH 7.0), 0.5 g NaCl $liter^{-1}$] and resuspended in U buffer [10 mM sodium phosphate (pH 7.0), 0.1 mM $MgSO_4$] into a turbidity at 600 nm of 2.5-3.0. The cultures were incubated at 30° C. with simazine at a final concentration of 0.06 mM. Culture medium without bacteria was used as a control. Samples were taken at 15 minutes intervals and these were centrifuged for 1 minute at 13,000 rpm. Simazine concentration was quantified by measuring absorbance at 225 nm using a spectrophotometer. The strain was able to degrade about 50% of the simazine compound

after one hour of incubation. FIG. **6** shows a simazine degradation kinetic of *Pseudomonas* sp. strain MHP41. Results shown were obtained from 3 independent experiments (FIG. **6**). In addition, the strain MHP41 grown in minimal medium using different nitrogen sources has the capability to degrade simazine Table 4).

TABLE 4

Table 4. Simazine degradation by *Pseudomonas* sp. MHP41 grown in minimal medium with different nitrogen sources.

Growth (Nitrogen Sources)	Simazine degradation
Ammonium	+
Nitrate	+ +
Urea	+ +
Cyanuric acid	+ + +
Simazine	+ + + +
Atrazine	+ + + +

Example 4

Viability and Simazine Degrading Ability of *Pseudomonas* sp. MHP41 in Sodium Alginate Matrix

[0068] A culture of *Pseudomonas* sp. MHP41 containing 1×10^8 CFU/ml (turbidity at 600 nm of 0.5) was encapsulated in 1% sodium alginate. The beads were stabilized in calcium chloride (9.3 g l^{-1}), kept in $CaCl_2$ and refrigerated at 4° C. Viability of encapsulated strain MHP41 was carried out according to the following procedure. 1 g of microcapsules was inoculated in minimal medium AM 0.4× with simazine 0.5 mM. Under this condition, the sodium alginate matrix is able to maintain its texture and integrity. Serial dilutions were carried out in 0.85% NaCl and were then plated in Trypticase Soy Agar (TSA) medium. TSA plates were incubated at 30° C. and *Pseudomonas* sp. MHP41 colonies were counted in the agar plates. Simazine was quantified by HPLC. At the beginning of the experiment, 1.2×10^7 CFU/ml was detected from the alginate beads. The number of viable cells increases during incubation time, reaching 3×10^9 CFU/ml after 72 hours. The encapsulation process with 1% sodium alginate allows the growth of *Pseudomonas* sp. MHP41, and do not affect its viability (FIG. **7**). For simazine quantification by HPLC, simazine was extracted from the culture media samples with methanol/water (80:20). The simazine degradation capability of *Pseudomonas* sp. MHP41 was not reduced by encapsulation in sodium alginate matrix. The simazine (0.5 mM) was removed from the liquid AM medium in less than 48 hours. Sodium alginate capsules without bacteria do not remove simazine from the medium. Results were obtained from 3 independent experiments (FIG. **7**).

Example 5

Bioremediation with *Pseudomonas* sp. Strain MHP41 of Simazine in Soil

[0069] The bioremediation experiments at the microcosms scale were performed to study simazine degradation in agricultural soil by the addition of the native s-triazine-degrading *Pseudomonas* sp. strain MHP41.

[0070] The strain was analyzed in two different types of soil: i) soil without history of simazine application and ii) soil annually treated with simazine. Soil was collected from the surface layer (0 to 15 cm), sieved through 5-mm, 2.8-mm and 2-mm mesh sieves and then contaminated with commercial simazine (Gesatop® 90WP). *Pseudomonas* sp. strain MHP41 was grown in minimal medium containing simazine as the sole nitrogen source and was added to the soil as immobilized inocula in sodium alginate matrix at a concentration of 1×10^8 CFU/g of dry soil (equivalent to a cell biomass of 1×10^{12} CFU/ml). Microcosms were inoculated each 4 days during four weeks. Soil moisture was controlled weekly. Soil samples were collected for microbiological and analytical determinations in triplicate. Soil samples were used to estimate the total heterotrophic bacteria count (THC) in trypticase soy agar plates (TSA, OXOID). The Most Probably Number (MPN) method was performed to estimate the simazine catabolic activity in the soil. Simazine was quantified by HPLC following extraction from soil with methanol/water (80:20) solution.

[0071] In soil previously not exposed to simazine, i.e. soil with no history of simazine application (FIG. **8**), bioaugmentation with *Pseudomonas* sp. MHP41 increased the total heterotrophic bacteria count and simazine catabolic activities. In none inoculated treatments, no active simazine-degrading microbial population was detected and no herbicide removal was observed. Bioaugmentation with *Pseudomonas* sp. strain MHP41 increased the simazine catabolic activity and attenuated the simazine concentration in the soil with a half-life of 15 days (FIG. **8**).

[0072] In soils with history of simazine herbicide application (FIG. **9**), the total heterotrophic bacteria count showed minimal changes between inoculated and none inoculated treatments. Furthermore, the simazine half-life in uninoculated microcosms was 11 days. When soils were bioaugmented, the simazine half-life was reduced to 3 days. Complete simazine attenuation in the inoculated treatment was observed after 24 days (FIG. **9**).

[0073] This example demonstrates that the native *Pseudomonas* sp. strain MHP41 increases the catabolic activity in both types of soil with different simazine application histories. Furthermore, the addition of this encapsulated strain attenuates the concentration of herbicide in both type of soils. Therefore it can be used for bioremediation strategies.

Example 6

Bioremediation of Simazine in Soil with Lyophilized *Pseudomonas* sp. Strain MHP41

[0074] To evaluate the simazine-degrading potential of *Pseudomonas* sp. strain MHP41 in agricultural soil, bioremediation experiments in microcosms were performed using lyophilized cells of the strain. Lyophilized cells of strain MHP41 were activated in liquid AM minimal medium and added to the soil following the same experimental set-up described in Example 5. Simazine attenuation was observed, and a decrease of about 50% of simazine was obtained in the soil during the first week of incubation.

[0075] This example, demonstrates that the lyophilized process does not affect the simazine-degrading ability of *Pseudomonas* sp. strain MHP41 in the soil.

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1. A bacterial strain able to degrade or mineralize s-triazines, corresponding to *Pseudomonas* sp. MHP41, deposited under the access number NRRL B-30908 on Mar. 17, 2006.

2. A product for the bioremediation of s-triazine-contaminated environments, including a bacterial inoculum of *Pseudomonas* sp. strain MHP41 (NRRL B-30908), according to claim **1**, and a culture medium, a buffer solution or sodium alginate.

3. The product for the bioremediation according to claim **2**, wherein the inoculum contains *Pseudomonas* sp. strain MHP41 (NRRL B-30908) from 1×10^{3} UFC/ml to about 1×10^{15} UFC/ml of culture medium or buffer solution or from 1×10^{5}UFC/g to 1×10^{12} UFC/g of sodium alginate.

4. The product for the bioremediation according to claim **2**, including a bacterial inoculum of lyophilized cells of *Pseudomonas* sp. strain MHP41 (NRRL B-30908) in a sterile complex protective medium.

5. The product for the bioremediation according to claim **2**, including an inoculum of immobilized cells of *Pseudomonas* sp. strain MHP41 (NRRL B-30908) in sodium alginate.

6. A method for the bioremediation of s-triazine-contaminated environment, comprising the steps of:

a) adding the product according to claim **2**, to the environment contaminated with s-triazines, and

b) incubating the product, in the environment for a period of time sufficient to permit the complete degradation of the s-triazine compound in the environment, wherein the period of time ranges from at least 1 week to about 12 months.

7. A method for the bioremediation of s-triazine-contaminated environments according to claim **6**, wherein the period of time ranges from 1 week to 4 weeks.

8. A method for the bioremediation of s-triazine-contaminated environments according to claim **6**, wherein the concentration of *Pseudomonas* sp. strain MHP41 (NRRL B-30908) ranges from about 1×10^{2} CFU/g of soil to about 1×10^{12} CFU/g of soil.

9. A method for the bioremediation of s-triazine-contaminated environments according to claim **6**, wherein the s-triazine in the contaminated environment is selected from the group consisting of simazine, hydroxysimazine, deethylhydroxysimazine, atrazine, hydroxyatrazine, deethylatrazine, deethylhydroxyatrazine, deisopropylatrazine, fluoroatrazine, propazine, terbutylazine, cyanuric acid and cyanazine.

10. A method according to claim **9**, wherein the s-triazine is a molecule that contain a chlorine atom.

11. A method according to claim **10**, wherein said s-triazine is simazine.

12. A method according to claim **10**, wherein said s-triazine is atrazine.

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