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Molecular characterisation of the bacterial diversity potentially degrading triclosan present in the Xichú river basin, Guanajuato

Caracterización molecular de la diversidad bacteriana potencialmente degradadora de triclosán presente en cuenca del rio Xichú, Guanajuato

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Abstract

Triclosán (TCS), is a trichlorinated phenoxyphenol having antibacterial properties. The aim of this study was to molecularly characterize bacterial diversity with potential to degrade Triclosan in isolated samples Xichú River in the Biosphere Reserve Sierra Gorda of Guanajuato. For molecular characterization of bacteria-based amplification techniques 16SrRNA gene sequences were used. The amplification products were purified and analyzed and compared sequences in databases. These affiliations allowed to infer phylogenetic relationships among prokaryotes. The analysis shows that the microbial diversity with potential to degrade triclosan is dominated by members of the genus *Bacillus* belonging to fermicute taxa, and to a lesser extent *Aeromonas* belonging to taxa Gama-proteobacteria, both groups considered as potential organisms for bioremediation sites contaminated. This work is the first report documenting the molecular characterization of bacteria with the capacity to resist and degrade Triclosan in Guanajuato. These data are of great value when implementing future technologies for the remediation of contaminated in the field of bioremediation environments.

Triclosan, Emerging contaminants, Bacterial diversity, Biodegradation

Resumen

El triclosán (TCS), es un fenoxifenol triclorado con propiedades antibacterianas. El objetivo de este estudio fue caracterizar molecularmente la diversidad bacteriana con potencial para degradar Triclosán en muestras del Río Xichú en la Reserva de la Biosfera "Sierra Gorda" de Guanajuato. Para la caracterización molecular se utilizaron técnicas basadas en la amplificación de secuencias del gen 16SrRNA. Los productos de la amplificación fueron purificados y las secuencias analizadas y comparadas en bases de datos. Estas afiliaciones permitieron inferir relaciones filogenéticas entre los organismos. El análisis muestra que la diversidad microbiana con potencial para degradar triclosán es dominada por el género *Bacillus* que pertenecen al taxa fermicute, y en menor proporción el género *Aeromonas* perteneciente al taxa gamma- proteobacteria, ambos grupos considerados como organismos potenciales la biorremediación de sitios contaminados. Este trabajo es el primer reporte que documenta la caracterización molecular de bacterias con capacidad para resistir y degradar Triclosán en Guanajuato. Los datos son de gran valor a la hora de implementar futuras tecnologías para la recuperación de ambientes contaminados en el ámbito de la biorremediación.

Triclosán, Contaminante emergente, Diversidad bacteriana, Biodegradación

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Introduction

In the last decades, among the organic pollutants in water, so-called emerging pollutants have been increasingly detected, which correspond in most cases to unregulated pollutants, which may be candidates for future regulation, depending on research on their potential health effects and monitoring data regarding their occurrence. Pharmaceuticals used in humans and animals have been identified as emerging environmental contaminants (Daughton, 2004).

The list of new pollutants includes analgesics, anti-inflammatory drugs, anti-epileptics, blockers and antibiotics, and even personal care products such as soaps, toothpastes, and deodorants among others, which are discharged into sewage systems. The presence of these emerging pollutants in municipal effluents has been dispersed into the environment causing a negative impact on both health and ecosystems (Ternes et al., 2004). Some of these compounds can alter the endocrine system by blocking or disrupting functions in humans and animals even when found at exceptionally low concentrations (García-Gómez et al. 2011). Recent studies show that it is increasingly common to find concentrations of nanogram to micrograms per litre for many of these products in wastewater, making treatment techniques for these compounds insufficient (Vienoa et al. 2007). A clear example of emerging contaminants is Triclosan, which is used as a bactericide. This emerging contaminant has no industrial regulation and is dispersed in the environment, affecting the lives of different organisms.

The suggested mechanisms by which TCS is removed from surface waters are biodegradation, photolysis, chlorination and association with surface solids. Since conventional methods have not shown convincing results for the removal of these pollutants, biodegradation emerges as an attractive alternative. In that sense, the search for triclosan degrading microorganisms is a tool that can be used in biological digestion to complement and/or increase the effectiveness in the removal of triclosan and other persistent pollutants.

Literature review Triclosan

Triclosan [5-chloro-2-2,4-dichlorophenoxyphenol], TCS, is a trichlorinated phenoxyphenol, with the chemical formula $C_{12}H_7Cl_3O$, has a molecular weight of 289,546, and a function point of 55-57 ℃, is a potent antibacterial and fungicidal agent. It is a colourless solid with a slight phenol odour. It is a chlorinated aromatic compound which has functional groups representative of ethers and phenols. Its solubility is, 0.01 g/L for water; 0.1 N NaOH, 23.5 g/L; ethanol, and in acetone it is highly soluble (FDA, 2008).

Triclosan is present in many disinfection-related products such as soaps, deodorants, cleansers, shampoos, and cosmetics. In addition, it is suitable for introduction into polymers and fibres, mattress pads, cutting boards, shoes, and sportswear (Glaser A; 2004).

Triclosan transformation products and related compounds

Due to their reactivity, their main transformation products as hydrolysis products of triclosan in aqueous media are the chlorophenols: methyltriclosan, 2,4-dichlorophenol, 2,3,4-trichlorophenol and 2,4,6-trichlorophenol.

Dioxins are one of the most dangerous products of triclosan photodegradation, can be highly carcinogenic and can cause such serious health problems as suppression of the immune system, decreased fertility, disruption of sex hormones, birth defects, miscarriages, and cancer. TCS is listed as a "possible" precursor contaminant of dioxins (Latch, et al, 2005).

Antibacterial properties

Triclosan is a phenolic derivative that at low concentrations inhibits essential enzymes of metabolism or binds to essential cell wall metabolites, causing wall degradation in some groups of bacteria. It exhibits bactericidal properties on Gram+ and Gram- bacteria as well as fungi and yeasts. (Canosa-Rodríguez, 2009)

At high concentrations it causes cell lysis and inhibits the enzyme enoyl-ACP (acyl carrier protein) reductase which is involved in fatty acid synthesis (Trilla, 2005).

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Environmental problem of Triclosan: Sources of contamination

Its production began in the 1970s for personal care, as a control of bacteria that could affect human health.

Between 1976 and 2010, the US Patent and Trademark Office issued more than 2,900 patents containing the word "Triclosan".

As it is present in multiple products related to disinfection such as veterinary products, medicines, cosmetics, fragrances etc. The main sources of environmental contamination by triclosan considered according to Dann and Hontela (2011) are domestic wastewater discharges as well as incomplete removal of triclosan in wastewater treatment plants, allowing its distribution on soil and water surfaces.

In different countries around the world, including Mexico, triclosan has been detected in treated and untreated water, as well as in effluents and effluents from lakes, rivers, and seawater among others (Canosa-Rodriguez, 2009).

Effects of Triclosan

One of the biggest problems arising from the introduction of triclosan into the environment is the adverse effects it can cause. The main affected by this problem are aquatic flora and fauna, such as unicellular algae and cyanobacteria. According to a study by Orvos in 2002, these unicellular organisms suffered growth inhibition using Triclosan concentrations of between 1.3 and 13 ng/mL. About amphibians, in the species *Rana pipiens* (leopard frog) it was observed that the organism lost weight, as well as a high mortality rate at Triclosan concentrations of 230 ng/L (Fraker, 2004).

For the species *Rana catesbiana* (bullfrog), thyroid hormone-mediated changes in their premature metamorphosis have been observed, affecting their phenotype as an increase in tadpole tail size and decrease in weight using a concentration of 150 ng/mL (Veldhoen, 2006).

Reports for mammals state that in rats Triclosan decreases thyroid hormone levels but the concentrations needed to observe these changes are higher for aquatic organisms as 30 mg/kg per day is necessary (Crofton, 2007).

Triclosan degradation processes

Because Triclosan is dispersed in the environment and difficult to remove by conventional methods, several mechanisms of Triclosan degradation have been studied. One alternative is advanced oxidation processes (AOP), where the use of different photocatalysts such as hydrogen peroxide, ozone, and metal oxides of zinc and titanium have been investigated, the results have been relatively good at low concentrations, some methods use Pd/Fe nanoparticles, photodegradation, even ultraviolet radiation and free chlorine (Molina, 2014). In the case of free chlorine, degradation varies according to the pH of the sample; the more neutral the pH, the more effective the degradation will be (Canosa et al; 2005).

Other little studied methods are the biodegradation of Triclosan with bacteria. Recently it has been found that some bacteria are able to resist this compound and even degrade it, some of the genera of these bacteria are Pseudomonas (Molina, 2014) and Achromobacter xylosoxidases (Canosa, 2008).

These resistance capabilities have been documented for two types: Intrinsic and Acquired which arise by mutation or by the acquisition of genetic material in the form of plasmids or transposons; these configurations allow large arrays of resistance genes (Cabrea et al; 2007).

Other wastewater microorganisms that have been documented to have the ability to degrade triclosan include *Sphingomonas* spp. Rd1, *Nitrosomonas europaea*, *Sphingomonas* spp. PH-07 and *Sphingophyxis Strain* KCY1 (Hay et al. 2001, Roh et al. 2009, Lee et al. 2012 in Lee, et al., 2013).

In most cases, treatment to remove a compound does not necessarily involve mineralisation, so the likelihood is that the parent compound has been transformed, changing its functionality and toxicity.

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In the case of treatments with microorganisms, the problem lies in the pathogenicity of most of the microorganisms studied. On the other hand, the studies carried out to determine the presence of triclosan-degrading microorganisms in Mexico are quite scarce, without specifying the specific basins investigated.

It is necessary to use new technologies aimed at solving this problem and guaranteeing the complete elimination of these pollutants, which can have serious consequences on the health of human beings, flora, and aquatic fauna in the effluents where they are discharged.

Analysis of bacterial diversity

Using molecular techniques using phylogenetic markers such as the 16S ribosomal gene, genomic libraries have been constituted whose members of different groups and sub-groups include in their order: Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and Firmicutes (as the most abundant), (Forney, 2004).

Our working group has recently reported physicochemical and microbiological parameters of water quality, as well as the isolation of triclosan resistant and degrading microorganisms in the micro-watershed of the Xichú river and its intersection with the Laja river in the hydrological sub-watershed of the Santa María river in the Sierra Gorda Biosphere Reserve of Guanajuato (González C. et al, 2014).

In this context and given the importance of the use of microorganisms in biodegradation processes, this work proposes to contribute to the identification and molecular characterisation of the bacterial diversity involved in the degradation of this type of pollutants.

Methodology

Microbial resources

The bacterial strains were previously collected from water bodies in the state of Guanajuato, obtaining five strains from the municipality of Xichú, Gto. with the key M1SA, M310-3, M4AA, M1Sp and M110-3ª (González C., et al., 2014) and one strain from the municipality of Yuriria, Gto. with the key E4. (Rodríguez-Rodríguez, et al., 2013) for a total of 6 strains selected for their ability to use Triclosan as a carbon source.

DNA extraction

Bacterial isolates were inoculated in liquid LB medium (5 ml) for 48 hr. A pellet is obtained after centrifugation at 110 rpm. Lysis was carried out in 300 µl of buffer (TRIS- EDTA) and vortexed for 1 min followed by mechanical lysis with glass beads (Atashpaz et al; 2010). Subsequently 10% SDS was added and subjected to shaking. Then 50 µl NaCI (5 M) and 50 µl LiCL (5 M) were added and allowed to stand for 5 min at room temperature. 5ul RNA was added to each tube. They were then incubated at 37°C for 10 min. (Eguiarte et al; 2007). 500 µl of the supernatant was taken and a volume of phenol-chloroform was added to the samples. 400 µl of the aqueous phase was taken and 2 volumes of absolute ethanol were added with vigorous shaking. Finally, the genetic material pellet could dry. Finally, it was suspended in 50 µl of sterile water, shaken gently to dissolve and left to freeze at -20°C (Zavala, 2005).

Horizontal agarose gel electrophoresis

DNA visualisation was performed in a horizontal electrophoresis chamber on 1% agarose gels in TAE 1X buffer. 2µl of DNA extraction sample and 1µl of GelGreen loading buffer were loaded, mixed and placed in the wells. Finally, the chamber was set to 100 volts for 30 min. The molecular marker was placed in the last well. The displacement of the samples was observed through a transilluminator. The image was transferred to a PC.

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Amplification of the bacterial 16S rRNA gene by polymerase chain reaction (PCR)

For the amplification of the 16S rRNA genes by PCR technique, the universal primers F27 and R1492 (Wang, 1996) specified in Table 1 were used. 25 µl of 2x Dream Taq Mix, 1µl of the universal primers F27, 1µl of DNA sample and 22µl of sterile water were placed in an ependorff tube, thus obtaining a volume of 50 µl.

Indicator code	Sequence (5´- 3´)	Size
F27	AGAGTTTGATCMTGGCTCAG	20
R1492	TACGGYTACCTTGTTACGACTT	22

Table 1 Initiators Used for Amplification and Sequencing

They were then placed in the Multigene thermal cycler with the following amplification conditions: First stage at 95 °C, 5 min, which at 35 cycles as shown in Table 2.

	(1 cycle)		(35 cycles)		(1 cycle)	
Temp.	105°C	94°C	94°C	53°C	72°C	4°C
Time	3:00 min	0:50 min	1:00 min	1:30 min	5:00 min	∞

Table 2 Temperatures and times used in the Polymerase Chain Reaction process at a volume of 50µl

This amplification process was carried out twice to ensure a significant sample in the purification process, thus obtaining a total volume of 100 µl per sample. The PCR products were then subjected to electrophoresis in a 1% Agarose gel for 30 minutes at 80 volts.

Purification of Amplification Products

The purification of the genetic material was carried out with the Zymo DNA Clean & Concentrator™-25 Purification Kit (Zymo Research) obtaining a total volume of 40 µl per sample. Finally, electrophoresis was performed by placing 2 µl of the purified sample and 1µl of GelGreen to observe the purified sample.

16S Ribosomal Gene Sequencing

After purification, approximately 38 µl of each sample was sequenced at the Cinvestav-Langebio Genomic Services Department using a Sanger sequencing process.

Molecular analysis of the 16S ribosomal gene of the bacterial diversity with potential for Triclosan degradation.

The last step was the comparison of the sequences obtained with those deposited in the databases. The first step was to use the Blast software of the NCBI (National Center for Biotechnology Information) website to compare the sequences with those of this database. In the next step, the GreenGen database was used, which contains only bacterial sequences of the 16s gene, with the aim of downloading type sequences to observe the similarities between groups, at this point the type sequences of the species with affinity were downloaded. Finally, the 6 sequenced samples and the type sequences were subjected to a maximum likelihood analysis in the MEGA software for the elaboration of a phylogenetic tree, thus observing the phylogenetic relationships between each group.

Results and discussions DNA extraction and visualisation

The extraction process was essential for DNA purification and well defined bands were achieved as shown in Figure 1. The amount of DNA was sufficient and reliable for the amplification process shown below. From the DNA integrity analysis it is concluded that the samples present an acceptable concentration and purity for subsequent amplification.

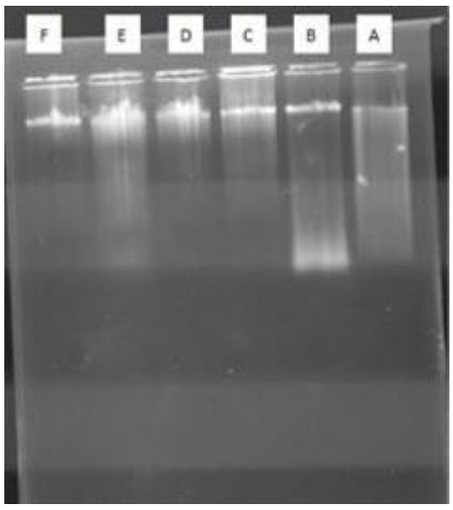

Figure 1 DNA integrity of Chromosomal DNA samples from bacterial strains with the ability to use Triclosan as a sole carbon source, A.- E4, B.- M1SA, C.- M310-3, D.- M4AA, E.- M1Sp, F.- M110-3

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PCR amplification products

Once the 16s rRNA gene was amplified to a volume of 50µl, an electrophoresis was carried out to determine if the observed bands were reliable for purification, the band corresponding to the amplification product of approximately 1500 bp was observed in the samples.

This process had to be repeated several times because not all amplifications were successful, even when the genetic material looked reliable, this is due to substances that interfere with the polymerase by partially or totally blocking its catalytic activity such as some salts (Newlester-Microbial 2009), a factor in reproducing the amplification was the required volume of 100µl of each sample. Figure 2 shows the PCR products for the amplification of the 16S rRNA gene of the bacterial samples M310-3, M4AA and M1Sp. In the other three samples, the 16s gene is not observed. Finally, after making the corresponding adjustments, the amplified products were obtained for samples M1SA and M110-3 and E4 (not shown).

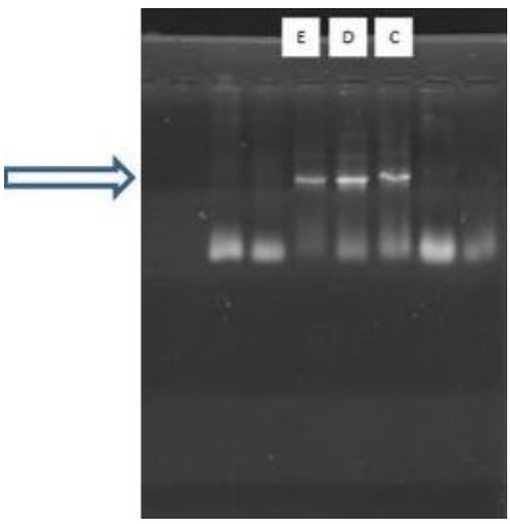

Figure 2 Amplification product of approximately 1500 bp C.- M310-3, D.- M4AA, E.- M1Sp

Purification of amplification products

Samples were purified by the Zymo DNA Clean & Concentrator™-25 Purification Kit. As a product of the purification two fragments are obtained for most of the samples as can be seen in figure 3, both fragments differ from the expected size of 1500 bp.

The results are reproduced by repeating the experiment, however, the purification method proved to be effective for sequencing the amplified fragment. A possible explanation could be interference in the gel run due to an overload of genetic material.

Sequence analysis and identification of Triclosan-degrading bacterial diversity

The edited sequences were run in the BLAST algorithm of NCBI, to determine the degree of homology of the sequences of the isolated strains with respect to the type sequences deposited in the GreenGen database, which contains only bacterial sequences of the 16s gene, twelve type sequences of several species of the genus *Bacillus* and the genus *Aeromonas* were downloaded. The 6 sequenced samples and the twelve type sequences were subjected to a maximum likelihood analysis in MEGA software for the elaboration of a phylogenetic tree (Figure 4) observing the phylogenetic relationships between each group. Where sample E4, M1AA and M110-3 have an affinity with B. *safensis* and pumilus, while sample M1Sp has a phylogenetic relationship with *B. subtilis*, sample M310-3 has a strong resemblance with B. cereus and thuringiensis, and finally sample M1SA has a strong phylogenetic relationship with *Aeromonas hydrophila*.

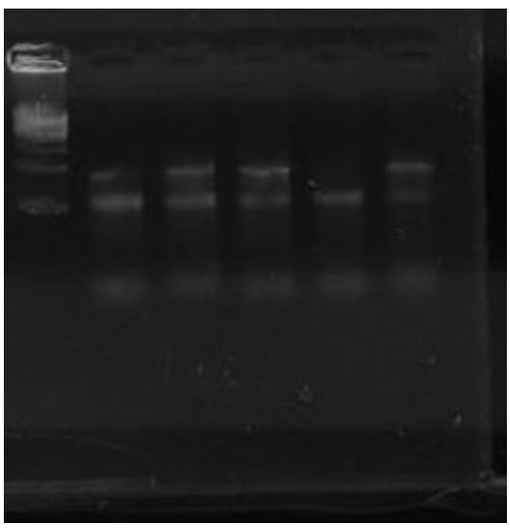

Figure 3 1% Agarose Gel purified amplification fragments, lane 1 molecular marker 1kb (left) lanes 2-6 Samples E4, M1SA, M310-3, M4AA, M1Sp

ISSN-On line: 2414-8830

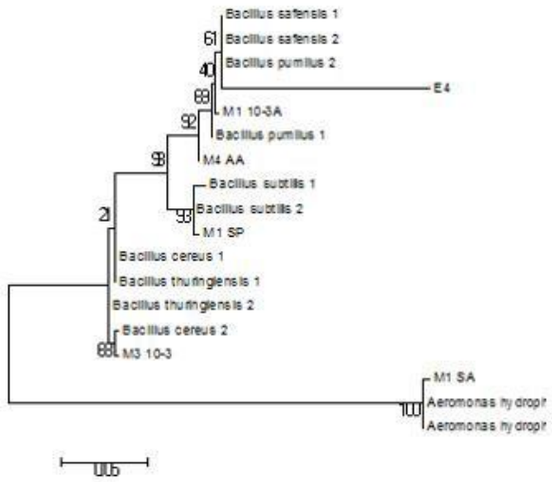

Figure 4 Phylogenetic tree made in MEGA, showing the phylogenetic relationships between the sequenced samples and the type sequences.

The microorganisms of the genus *Bacillus* are large (4-10 µm), Gram-positive, strict aerobic or encapsulated facultative anaerobic bacilli.

An important characteristic is that they form spores that are extraordinarily resistant to unfavourable conditions (Bartram et al; 2003), which may be a key feature for their ability to grow in contaminated water bodies and/or in the presence of Triclosan. B. safensis and pumilus bacteria are closely related based on phenotypic characteristics and 16s sequences (Branquinho et al; 2007), both species are reported to be found in industrial wastewater environments (Satomi, 2006), which could be another indicator for Triclosan resistance. However, *B. pumilus* species have been found in food-borne infections and even skin infections in humans (Bentur, 2007), which represents a more sensitive handling for this species.

B. subtilis species are generally found in sediments and in the rhizosphere and produce heat resistant endospores and have a resistance to chemical disinfectants (Cuervo, 2010) and possibly gaining resistance and degradation to Triclosan. B. cereus is a food pathogen and is closely related phylogenetically to *B. thuringiensis* and this species can be differentiated from *B. cereus* because it produces crystals inside its cell during sporulation, however there is no report that *B. thuringiensis* has a parasitic life cycle, both have been isolated from water bodies as they are found in almost any environment (Perez, 2005) and it can be deduced that they would be found in contaminated sites.

For *Aeromonas hydrophila*, the characteristics of the genus refer to short Gram-negative bacilli between 0.3-1.0 µm (Altweeg, 1999), which are pathogenic organisms of reptiles, but there are reports of skin infections and diarrhoea in humans.

The life cycle is aquatic and previous isolates have been reported from sewage and chlorinated water (Kühn et al; 1997), thus possibly providing the ability to degrade to Triclosan.

All the bacterial strains found could have developed resistance to Triclosan thanks to intrinsic or acquired capacities and which could be indicators of contaminated water as they are resistant to several contaminants thanks to their previous isolations in contaminated water which has given them this resistance to the compound Triclosan, however, more studies are needed on the degradation pathways that these strains possess.

Conclusions

Six potentially Triclosan-degrading bacterial strains were found, of which five belong to the genus Bacillus and one to the genus Aeromonas. The characterised bacteria could be used to degrade Triclosan in further studies due to their biodegradation potential. However, it is necessary to consider that some species are classified as pathogenic and care should be taken in their handling, such as B. cereus and Aeromonas.

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