Detection of integron-encoded integrases in environments with moderate anthropogenic impact from Puerto Rico

By

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Abstract

Integrons are genetic elements with a site-specific recombination system which includes an integrase (IntI) capable of capturing and expressing promoterless, circular DNA molecules known as gene cassettes. Although integrons were initially discovered as elements involved in the transfer of multiple antibiotic resistance mechanisms within the clinical scene, their presence has been increasingly reported in environments with other integrated adaptative traits unrelated to antibiotic resistance functions. This project explores the presence of integrons in microbial communities from environments with moderate anthropogenic impacts in Puerto Rico. These include two extreme environments: hypersaline waters from Cabo Rojo and the Coamo Thermal Springs and soil from the Culebrones Cave in Arecibo. Samples from these places were analyzed to determine the presence of integrases by culture-dependent and culture-independent techniques. Metagenomic DNA was analyzed with an *int*-targeted PCR assay using degenerate primers. Alignments were performed for all sequences recovered and their relationship with other tyrosine recombinases was analyzed. Sequences were also compared against functional integrases for their characterization. Important motifs characteristic of the tyrosine recombinase family such as: Patch (II and III) and Box (I and II) were detected. Twenty eight novel integrase sequences were recovered from metagenomic DNA extracted from hypersaline water and thirty integron integrases from cave soil. Additionally, eight new integron−encoded integrases were recovered from the Coamo Thermal Springs**.** Furthermore, integrase genes similar to integrase class 2 that do not contain the premature stop codon X179 were detected by PCR in two halophilic isolates closely related to the genera *Salinivibrio* (γ-*Proteobacteria*) and *Bacillus*. One isolate from the Coamo Thermal Springs related to the genus *Geobacillus* showed integrase genes similar to integrase class 1. This study provides the first evidence of the presence of inte-

grase integrons among the microbial community found in environments with moderate anthropogenic impacts from Puerto Rico and in the Caribbean. The results obtained also support the argument that the integron-based mechanism for horizontal gene transfer is widespread and can take place in solar saltern ponds, Coamo Thermal Springs and cave soil.

Resumen

Los integrones son elementos genéticos con un sistema de recombinación especifica el cual incluye una integrasa (IntI) capaz de capturar y expresar moléculas de DNA circulares y sin secuencias promotoras, conocidas como genes casetes. Aunque los integrones fueron inicialmente descubiertos en ambientes clínicos como elementos envueltos en la transferencia de múltiples mecanismos de resistencia, se ha reportado su presencia en ambientes con otras funciones adaptativas no relacionada a funciones de resistencia a antibióticos. Este proyecto explora la presencia y diversidad de integrones en comunidades microbianas de ambientes con impacto antropogénico moderado en Puerto Rico. Este incluye dos ambientes extremos las aguas hipersalinas de Cabo Rojo, los baños termales de Coamo y el suelo de Cueva Culebrones en Arecibo. Las muestras de cada lugar fueron analizadas para determinar la presencia de integrasas de integrones por técnicas dependientes e independientes de cultivos. Se analizó ADN metagenómico mediante un PCR especifico para *Int* utilizando cebadores degenerados. Se realizaron alineamientos para todas las secuencias recuperadas y se analizó su relación con otras recombinasas de tirosinas. Las secuencias fueron comparadas contra otras integrasas para su caracterización. Importantes "motifs" característicos de la familia de las tirosinas recombinasas fueron detectados como lo son: "Patch" (II y III) y "Box" (I y II). Veintinueve secuencias de integrasas fueron recolectadas a partir de ADN metagenómico extraído de las aguas hipersalinas y treinta integrasas de integrón del suelo de Cueva Culebrones. Ocho secuencias de integrón integrasas fueron recolectadas desde los Baños Termales de Coamo. Genes de integrasa relacionada a integrasa clase 2, que no contienen el codon de terminación prematuro X179, fueron detectados por PCR en dos aislados halofílicos relacionados al género *Salinivibrio* (γ-*Proteobacteria*) y *Bacillus*. Un aislado desde los Baños Termales de Coamo relacionado al género de *Geobacillus* presentó ge-

nes de integrasas similares a los de la integrasa clase 1. Este estudio provee la primera evidencia de la presencia de integrón integrasas entre comunidades microbianas encontradas en ambientes con impacto antropogénico moderado de Puerto Rico y el Caribe. El resultado obtenido, además, apoya el argumento de que los mecanismos de integrón para la transferencia horizontal de genes es esparcido y puede tomar lugar en ambientes halofílicos, termofílicos y en suelo de cuevas.

Dedication

Several important people are part of the success of my research and I want to dedicate this thesis to them, my Family. They are the most important people in my life (Biky, Mom, Dad, Natalia), without their help and support I would not be the human that I am now. They were my inspiration for reaching my goal because they believed in me and in the hard moments they were always there to give me support and advice. Another important person I want to dedicate my thesis to is Josué Dumeng Valentín. He provide me peace and calmness during the last hard moments and frustration through love and patience.

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Introduction

Integrons are genetic elements encoding a site-specific recombination system capable of capturing and expressing promotorless DNA units, called gene cassettes (Hall 1989, Mazel 2007; Rodríguez−Minguela et al., 2009; Stokes 1989). Most of the integron-related studies focus their attention on hospital environments since they were discovered within the context of the antibiotic resistance problem. Fecal and industrial effluents have been linked to the presence of integron-encoded antibiotic resistance in environmental settings (Rodríguez-Minguela et al. 2009; Elsaied et al., 2011). However, a worldwide scale study revealed that integrase from clinical integrons are not resctricted to the hospital scene (Rodríguez-Minguela et al. 2009). Integrons found in natural environment are important since they can be associated with the acquisition of new adaptive functions and the establishment of antibiotic resistance reservoirs (Koenig *et. al.*, 2008; Stokes et al. 2001). However, there are few reports on the presence and role of integrons in extreme environments. Some of these reports include studies of the metagenome associated with uranium mines and deep sea hydrothermal vents, which revealed the presence of gene cassettes potentially encoding adaptative functions unrelated to antibiotic resistance genes as well as new integron integrase sequences (Ghauri et al. 2003; Elsaied et al. 2007).

So far, no studies concerning the diversity and distribution of integrons have been conducted in extreme environments across the Caribbean region. The present study was focused on determining the presence of integron integrases in solar salterns, thermal springs and cave ecosystems located at Cabo Rojo, Coamo and Arecibo, respectively. This was achieved by using PCR−based methods with metagenomic DNA retrieved from the above mentioned sites and with genomic DNA from three halophilic and thermophilic isolates related to the genus *Salinivibrio* (γ-Proteobacteria), *Bacillus* and *Geobacillus.* This information will provide further insight on

the distribution of the module and its potential for driving evolutionary change and adaptation in extreme environments.

Literature review

I. Mechanism of horizontal gene transfer

Horizontal gene transfer (HGT) is the flow of genetic information across species and between bacteria, HGT has played an important role in bacterial evolution, adaptation into communities (Sobecky and Hazen 2009: van-Elsas and Bailey 2002), and also has significantly contributed to the aggravation of the antibiotic resistance problem in the absence and presence of selective pressure (Figure 1.1). Recently, HGT has been implicated in the dispersal of genes involved in cellular processes such as photosynthesis (Igarashi et al., 2001), nitrogen fixation (Sullivan et al., 2001**)**, carbon fixation (Utaker et al., 2002), sulfate reduction (Friedrich 2002), and pathogenicity (Hentschel, U., and J. Hacker, 2001; Gamieldien et al., 2002). Mechanisms for lateral-transfer between bacteria such as phages (Jiang and Paul 1998) transposons, and plasmids (Sobecky et al., 1998) have helped to widespread resistance without antibiotic exposure.

Transduction is a mechanism where genetic material can be transferred horizontally from a bacterial cell to another through bacteriophages. In this process, the phage genome can be integrated into the bacterial chromosome provoding new phenotypic characteristics. The phage excision process is not always exact, therefore bacterial genes can be incorporated into the virus and when it infects another cell, those genes can be integrated into the host genome (Ochman et al. 2000; Toussaint and Merlin 2002). In transformation**,** free DNA can be transferred horizontally from cell to cell and through a recombination process is incorporated into the host genome. The conjugation process is perfomed throught a sex pili where a conjugal tube is formed and a copy of single stranded DNA is passed to the recipient cell. The conjugation process is common in the intestinal tract of animals where bacterial populations are high (Garcia-Quintanilla, M., et al.,2008).

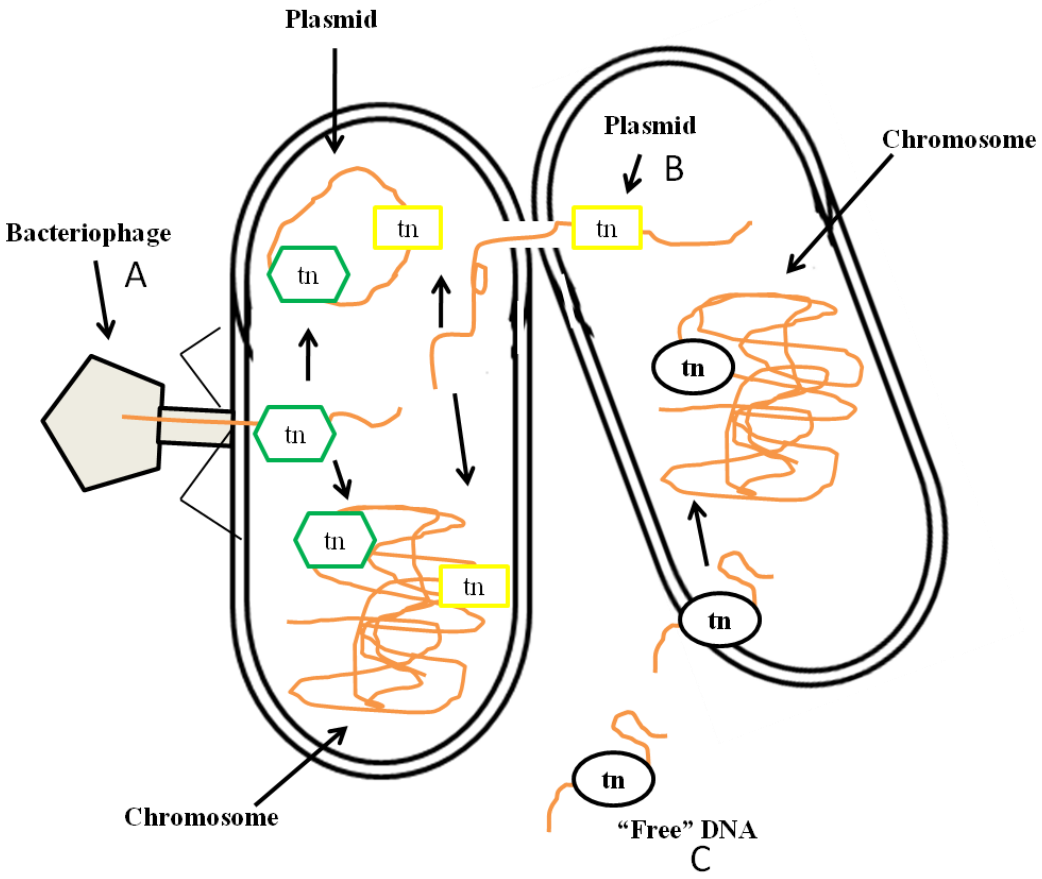


Figure 1.1 Different mechanisms of horizontal gene transfer are presented. A) Transduction: bacteriophages can be integrated into a bacterial chromosome providing new phenotypic characteristic to the cell; B) Conjugation: plasmid DNA is transferred from a donor to a recipient cell throught the use of a pilus: C) Transformation: cell-free DNA is taken and incorporated into the bacterial genome. Adapted and modified from Levy and Marshall, 2004.

II. Integrons

By the late 1980s Stokes and Hall identified the integron system as new type of mechanism in bacteria for the gain of genetic material encoding antibiotic resistance traits in bacteria. Integrons contain an integrase that mediates a site specific recombination reaction leading to the expression of open reading frames which generally lack promoters. Integrons integrases catalize the excision and rearrangement of resident cassettes (Stokes, H. W., and Hall, R.M. 1989). The gene that encodes the integrase is located at the 5' conserved region and it belongs to the superfamily of tyrosine recombinases. Members of this family are enzymes that perform site–specific

recombination and require short and specific DNA sequences involving the formation of a Holliday junction intermediate. This mechanism involves cleavage and rejoining of DNA strands without ATP utilization or DNA synthesis (Gopaul, D. N., and G. D. Van Duyne, 1999) The integrase nucleophile residue consists of a tyrosine (Tyr−302) located at the 5' conserved region and is responsible for the cleavage and formation of a covalent intermediate by esterification of a DNA 3' phosphoryl group (Grainge, I., and M. Jayaram**,** 1999: Mazel, 2006). The tyrosine recombinase family is characterized by the presence of functional residues within conserved motifs (boxes I and II and patches I, II and III). However, integron integrases relative to other tyrosine recombinases contain distinctive additional domain residues around patch III (Gravel, A., N. Messier, and P. H. Roy. 1998; Nield B.S., et al. 2001; Nunes−Duby, et al. 1998). In addition to the integrase gene, the 5' conserved region has also DNA sequences that act as insertion (att I) site for the transcription (Pc) of gene cassettes (Figure 1.2)**.** The integron integrase also recognizes a specific core sequence (GTTRRRYY) found at the 3' end of gene cassette in order to catalize the insertion.

Integrons class 1, 2 and 3 can be found in many gram-negative bacteria of clinical origin, specifically among members of the *Enterobactericiae* (Antunes, P., et al. 2006; Young-Kang H., et al., 2005; Martinez-Freijo, P. et al., 1998). Recently, integrons have been found in gram-positive bacteria such as *Corynebacterium glutamicum* (Fluit and Schmitz, 1999; Nesvera, J. et al., 1998) and *Sulfobacillus* genera (Ghauri, M.A., et al., 2003). The advance of genomics and the availability of numerous genome sequences from environmental bacteria allow to explain that integrons are ancient structures and widespread gene capture systems (Mazel, D., 2006).

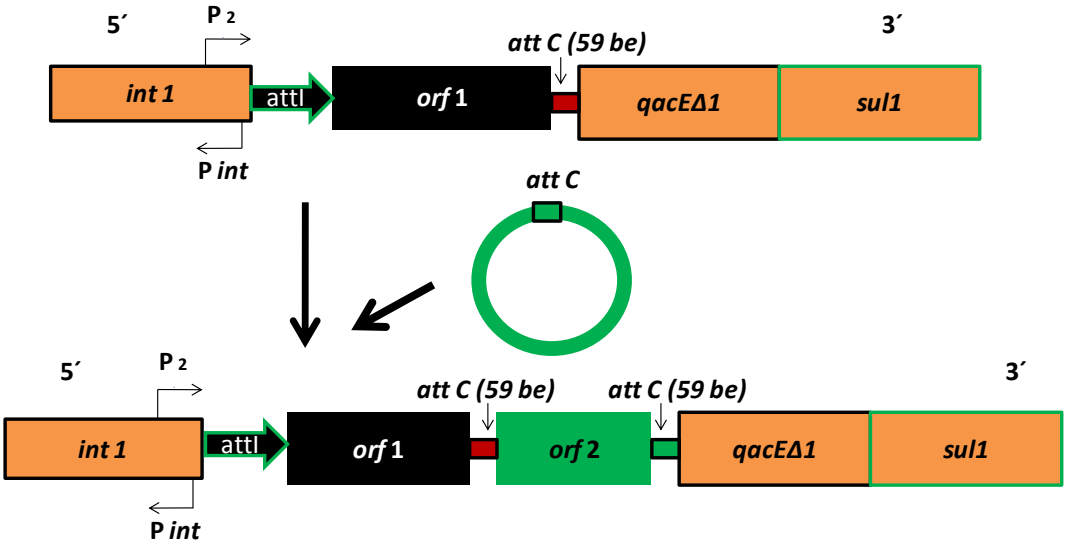


Figure 1.2 Representation of a class 1 integron and its integration mechanism. The region common to all integrons (5'-CS) generally consists of the following elements: an integrase coding gene (*intl*) the site-specific recombination sequence *attI* and two promoters, P, that drives the expression of integrase P(int) and the expression of the inserted gene cassette P(c). The 3'-CS is characteristic of class 1 integrons and contains permanent genes that confer resistance against quaternary ammonium compounds (qacE*Δ1)* and sulfonamide drugs (*sul1*). There is a second type of recombination site carried by gene cassettes that generally consist of a open reading frame (in this figure is *orf 2*)and presents at its 3' end a short imperfect inverted repeat sequence also called, *attC* which serves as a recognition site for the integrase (Collis, C.M. and Hall, R.M. 1992).

The integrons are divided into two different groups, although both contain similar organization: mobile integrons and chromosomal integrons differ. The mobile integrons are associated with transposons and plasmids, which are elements responsible for integron spread. The gene cassettes present in the mobile integrons are mainly involved in antibiotic resistance (Mazel, D, et al., 2010). Chromosomal integrons or superintegrons were discovered with a distinctive integrase named VchIntA which is located in the chromosome of *Vibrio cholerae* and was not associated with mobile DNA elements.

Recent studies had demonstrated that integron integrases are activated during SOS response, resulting in an increase of cassette rearrangements (Baharoglu, Z., et al., 2010). SOS is a bacte-

rial stress response which is induced as a consequence of abnormal rate of single stranded DNA (ssDNA) present in the cell, probably caused by UV radiation or exposure to antibiotics (Baharoglu, Z., et al., 2010). LexA is a repressor of the SOS response and the integron integrase RecA is an activator of both by stimulating the auto-proteolysis of LexA (Guerin, E., et al., 2009: Baharoglu, Z., et al., 2010).

III. Gene Cassettes

Gene cassettes exist as promotorless DNA molecules outside of the integron, in a closed circular form, and only are functional when inserted at specific locations in the host genome (defined by the presence of an integron) (Stokes, H.W. and Hall, R.M., 1989: Collis et al., 1993). The source of gene cassette remains unknown, but their expression is influenced by their position reactive to the Pc promoter. Their structure consists of a single open reading frame and short sequences of flanking DNA on both the 3' and 5' ends, called cassette attachment site (*attC*) or 59 base elements (59be). The attC sequence serves as integrase recognition site during the recombination event. These sequences vary in length from 54 bp to 141 bp, although there are two consensus regions. The core sequence found in the 5' and consist of the nucleotides GTTRRRY (R = purine; Y = pyrimidine) and the 3' inverted core sequences (ICS) consists of the nucleotides RYYYAAC (Figure 1.3). However, inverse core site in the *attI* site not is found therefore it cannot form a palindromic structure like *attC.* The open reading frame in the vast majority of the cases encodes a protein necessary for antibiotic resistance, however gene cassette encoding novel and unknow functions are being increasingly reported (Nield, S, et al., 2001). Recent studies suggests have shown other adaptative functions not associated with antibiotic resistance such as degradation of industrial wastes associated resistance to heavy metals, nitroaromatic catabolism, biosynthesis and DNA modification (Koenig, J. E., et al., 2009; Nemergut,

D.R. et al., 2004). Moreover, virulence factors, toxin-antitoxin systems and polysaccharide biosynthesis are associated with gene cassette functions (Ghauri, M.A., et al., 2003; Koenig et al., 2008).

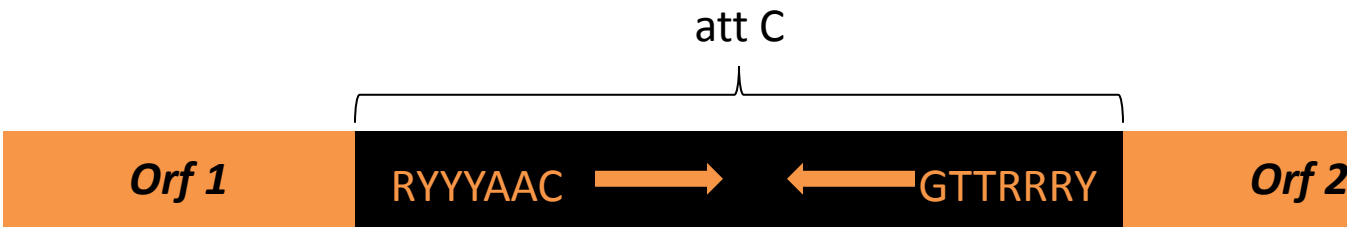


Figure 1.3 Representation of the core sequences found in the 5´ and the 3´ inverted core sequences (ICS) at the *attC* recombination site.

IV. Integron classification

Integrons are classified based on the difference in the percentage of identity of the integrase gene (Mazel, D., 2006) or their predicted amino acid sequences. The most common integrons found in many bacterial species are class 1 and 2 and are associated at clinical settings. Integrons class 4, 5, 6, 7 and 9 are associated with a novel class of integrons, called superintegrons.

Class 1 integrons are found in many pathogenic and commensal bacteria associated with humans and animals (Cambray, G., et al., 2010). Environments exposed to fecal contamination show prevalence for integrons class 1 and are typically associated to replicative Tn*21* transposons (Rodríguez, Minguela, et al., 2009). Also, they are the most widespread and studied of all integron classes. Two signature traits of class 1 integrons are the presence of *qacE*Δ and *sulf*1 genes at the 3'-CS region, which confer resistance to quaternary ammonium compounds and to sulphonamides drugs respectively Mazzel, D., 2006). However, there are reports where class 1 integrons have been detected lacking this typical 3'-CS region (Saenz, Y., et al., 2010).

Class 2 integrons are characterized primarily for the presence of an internal stop codon at amino acid position 179 in the *intl2* gene. Class 2 elements are mobilized by non replicative transposons *Tn7*. Recently, studies have described intI2 variants without the internal stop codon, in environmental samples (Rodriguez-Minguela, C, et al. 2009). In the terminal 3'-CS there are gene cassettes that conferred resistance against trimethroprim (*dfrAI*a o *dfrAI*b), streptothricin (*sat*) and streptomycin/spectinomycin (*aadA*1) (Sabaté, M. and Prats, G., 2002; Sundström, L., P., et al., 1998; Sundström, L. et al., 1991; Sundström, L. et al.,1990).

Classes 3 integrons are associated with transposons located into uncharacterized plasmids and are less frequently detected than class 2. Class 3 integrons had been described with gene cassettes encoding a metalobeta-lactamase in isolates of *Serratia marcescens* from Japan (Osano, E., et al., 1994). Two environmental strains, *Delfia acidovorans* C17 and *Delfia tsurruhatensis* A90 were found to contain class 3 integrons gene with gene cassetts encoding unidentified functions (Xu, H. et al., 2010).

Other integron classes include a class 4 integron identified in *Vibrio cholera* associated with mobile genetics element integrated in the chromosome via conjugation denominated, SXT. Also a Class 5 element found in compound transposon associated with a pRSV1 plasmid of *Alivibrio salmonicida* has been described (Mazel et al., 2010).

V. Super integrons or chromosomal integrons

Another group of distinctive integrons, called superintegrons or chromosomal integrons, have been found in many γ-*proteobacteria* such as *Vibrionaceae* and *Xanthomonads* (Cambray et al.,. 2010). Rowe-Magnus et al. 1999 postulate that the super-integron is an ancient element, possibly pre–dating the antibiotic era by thousands of years. These gene cassettes contain high similarity between their *attC* sequences and are located in the chromosome without association to

mobile elements. Class 4 was the first chromosomal superintegron (*Vibrio cholera*) and is known to contain 176 gene cassettes (Mazel et al., 1998; Rowe-Magnus et al., 2001). The largest superintegrons identified to date are in *Vibrio vulnificus* CMCP6 and YJO16; these are 153 and 138 kb long, and contain 219 and 188 gene cassettes respectively (Chen et al., 2003). It has been estimated that as little as five *Vibrio spp.* contain a gene cassette load equivalent in size to a small genome (Chen et al., 2003). Relative to clinical integrons, super−integrons contain a minor load of cassettes encoding antibiotic resistance; most of the genes associated with super−integrons encode functions unrelated to resistance traits (Rowe−Magnus, 2009).

Several superintegron cassettes contain homology to enzymes involved in energy metabolism and detoxification, such as 4-oxalocrotonate tautomerase which enables bacteria to use various aromatic hydrocarbons as their sole energy source (Whitman, 2002), a carbohydrate transport protein of the major facilitator superfamily, an adenylate kinase that interconverts ADP to AMP and ATP for driving energy requiring processes, and a lactoylglutathionelyase detoxification enzyme that converts toxic methylglyoxal (MG) to lactic acid (Ferguson et al., 1998). However, cassettes encoding virulence factors and DNA modification functions have been also described (Ogawa and Takeda, 1993).

VI. Integrons in extreme environments

Extreme environments are habitats distinguished by physical parameters such as salinity, temperature, pH, pressure, and oxygen concentrations that are extreme in relation to those present where eukaryotic organisms preferentially live (Stetter, 1998; Konings et al., 2002). Microorganisms found in extreme environments have considerable industrial application and potential for biotechnology development. Enzymes produced by extreme microorganism have unique features to tolerate extreme conditions such as high temperatures, extreme pH values, high pressure

and high salt (Van der Burg, 2003). However, the distribution and diversity of the integron platform across these extreme habitats remain relatively unexplored.

In genomic studies of halophilic bacteria, *Salinibacter ruber* demonstrated the ability to conduct lateral gene transference with halophilic members of the Archaea domain through transposons and possible presence of a integron integrase class 1 (Mongodin et al., 2005). Additional studies in man made salt mines were preformed in Pakistan where different gene cassettes were recovered from halotolerant strains related to *Halomonas magadiensis*, *Virgibacillus halodenitrificans* and *Yania flava*, and from metagenomic samples (Ghauri et al., 2006). The possible functions of these gene cassettes were associated with protein families of putative transcriptional regulators and a putative betaketoacyl reductase.

Integrons have been reported among acidophilic moderate thermophilic bacteria related to *Sulfobacillus thermosulfidooxidans* recovered from uranium mines, as well as in strains isolated from deep−sea hydrothermal vent (Ghauri et al., 2003: Elsaved, et al., 2007). Class 1 integron has been characterized in a thermophilic *Campylobacter* (O'Halloran et al., 2004). Moreover, integron integrases were detected in the acidophilic bacterium *Acidithiobacillus ferrooxidans* whereas 14 novel integron integrases and new gene cassette implicated in the metabolism of nitroaromatic compound have been detected in metal−contamined mine tailings (Rowe-Magnus and Mazel, 2001; Nemergut et al., 2004).

To date, no studies concerning the diversity and distribution of integrons platforms have been conducted from environments with moderate anthropogenic impact and extreme environments in Puerto Rico. The present study focused on determining the presence of integron integrases in Solar Saltern Ponds, thermal springs and a cave ecosystem located at Cabo Rojo, Coamo and Arecibo, respectively.

Objectives. Explore the presence and diversity of integron−encoded integrases in environments with moderadte anthropogenic impact using culture-independent and culture-dependent techniques; the two major objectives were the following:

1. Determine the presence and diversity of integrin encoded integrases by constructing and analyzing clone libraries of PCR-amplified metagenomic DNA.
2. Determine the presence of integrin encoded integrases via PCR from strains isolated from the Solar Saltern Ponds (Cabo Rojo), and Coamo Thermal Springs (Coamo).

Part I. Sampling Procedure

Material and Methods

I. Sampling sites: Description of the Solar Salterns in Cabo Rojo, Coamo Thermal Springs in Coamo, and Culebrones Cave in Arecibo, Puerto Rico.

1.1 Solar Saltern Ponds

The solar saltern are located on the southwest coast of Cabo Rojo. This system is a thalassohaline extreme environment which includes an estuary surrounded by natural mats, a mangrove forest, artificial ponds and saline lagoons (Fraternidad and Candelaria) with minimal exposure to anthropogenic impact (Fig. 1.4). The principal reason for this is due to its designation as a National Wildlife Refuge by the U.S. Fish and Wildlife Service since 1974. The Candelaria Lagoon is connected to the sea through a channel which we denominated: Puente. The solar saltern has limited contact with the sea and the constant high solar radiation together with the lack of rain during most of the year promotes the formation of a hypersaline environment (Departamento de Recursos Naturales, 1986). Additionally, the solar saltern are the most important stop site for migratory shorebirds in the Caribbean (U.S Fish Wildlife Service). Previously, researches have been studying the prokaryotic diversity of these salterns (Montalvo−Rodríguez et al., 1997, 1998, 2000), nevertheless no studies have been performed to determine the diversity and distribution of integron integrases.

1.2 Coamo Thermal Spring

There is at least one natural environment in Puerto Rico which can be considered alkalithermophilic. This habitat is the Coamo Thermal Springs located in the municipality of Coamo (south central Puerto Rico) which forms at the east-early trending of the Cordillera Central (Glover, 1971) (Fig. 1.4). Most of this area lies on the flank of a much-faulted geanticlinal volcanic core that is partly overlapped by a sequence of carbonate rocks and sediments (Glover, 1971). It is an unique thermal spring located on the island and its origin is not totally clear, although some theories about the origin do exist (Giusti, 1971). Until this moment, the most accepted theory is based on a geological fault in this area and the natural thermal gradient that exist on the terrestrial cortex (Quiñonez and Guzmán, 1983). Prokaryotic diversity of these thermal springs has been studied (Burgos, 2007), however no studies have been conducted with respect to detection of integron-encoded integrases.

1.3 Culebrones Cave

The Mata de Plátano Field Station is located 7 kms southwest of Arecibo, Puerto Rico, and inside of the reserve, a cave denominated Culebrones is found (Fig. 1.4). Culebrones Cave is a refugee for approximately 13 different bats species (Rodríguez-Durán and Soto-Centeno, 2003). Large numbers of native boas congregate to feed on bats. Culebrones Cave is considered a hot cave with temperatures ranging from 26−40ºC and relative humidity exceeding 99% (Rodriguez-Duran, 1998; Rodríguez-Durán and Soto-Centeno, 2003). Despite the ecological importance of cave ecosystems and their features, no studies have been performed in these environments regarding integrases integrons.

II. Sampling Sites.

To confirm our experimental hypothesis, in August 2008 different environments with moderate anthropogenic impacts in Puerto were analyzed to detect integron-encoded integrases. The environments analyzed with moderate anthropogenic impacts in Puerto include Culebrones Cave, Solar Saltern Ponds, Coamo Thermal Springs and Culebrones Cave. The samples were collected in duplicate using Whirl Pack®. Culebrones Cave soil samples were retrieved from two distinct points, soil at the entrance cave and soil within the cave. The temperature outside was 31.5 ºC and inside of Culebrones Cave was 38 ºC. The samples were stored at −20 ºC. In the case of Solar Saltern Ponds, water samples were collected from Candelaria and Fraternidad lagoons, artificial ponds (solar ponds) and ″puente″ (Table 1.1). The Coamo Thermal Springs water was collected from the mainstream where anthropogenic effect are minimal. Thermal water temperature was 39 ºC and pH was 8.9. Solar saltern (25 ml) and thermal springs (500ml) water samples were homogenized with a pipette following the filtration in 47mm, 0.45 μm (pore size) cellulose nitrate membrane filters (Whatman, Germany). Filtered membranes were stored at −20 ºC.

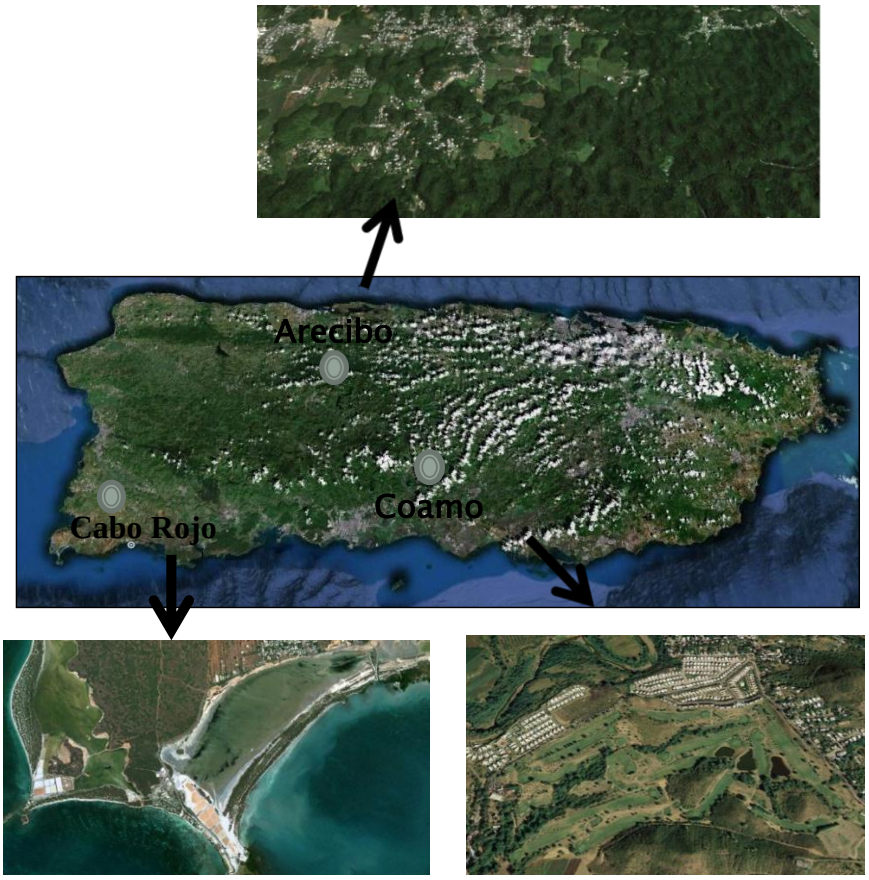


Fig 1.4 Location of sampling sites. Maps were obtained from Google Earth.

Table 1.1 Summary of physical parameters from the Solar Saltern Ponds (Cabo Rojo)

Sampling site	Temperature ºC	Salinity % w/v
Fraternidad	40	25
Candelaria	38	8
″Puente″	36	4
Solar ponds	40	35

Table 1.2 Summary of physical parameters from the Coamo Thermal Springs (Coamo)

Sampling site	Temperature ºC	pH
Mainstream	39 ºC	8.9

Table 1.3 Summary of physical parameters from the Culebrones Cave (Arecibo)

Sampling site	Temperature ºC	Humidity
Cave entrance (Cueva entrada)	31.5	90
Inside cave (Cueva adentro)	38	80

III. Metagenomic DNA extraction:

Metagenomic DNA extractions from cave soil, solar saltern ponds and thermal spring water were performed using the MoBio DNA extraction kit ® according to manufacturers instructions. Modification were performed to cause cell lysis; beat beading technique was used through vortex for 5 minutes at maximum speed followed by centrifugation at 10,000 rpm for 10 minutes. The isolated metagenomic DNA was resuspended in 50 µl molecular water and treated with RNAse (20 µg/µl) for 30 minutes at 37ºC. The DNA quality was verified on 0.8% agarose gels and staining with ethidium bromide. All metagenomic DNA's were used as templates for PCR amplification.

IV. PCR amplification of 16S rDNA and gel electrophoresis:

To prove the quality of the extracted DNA, the gene encoding the 16S rRNA was amplified by PCR using the forward primer Bact-27F and the reverse primer Bact-1392R (Table 2.1). The reaction mixture contained the following concentrations for a final volume 30 µl : 1X Buffer, 2.5 mM Mg, 1X BSA New England®, 2.5 mM dNTP's, 1 pMol of BACT 1392 and BACT 27, 0.026 U *Taq* polymerase (Promega) and 1 µl of template (15 ng/ µl). The PCR reaction consisted of initial denaturation of 5 minutes at 94° C; 30 cycles of denaturation 1 minute at 94° C; annealing 1 minute at 50 ° C and extension 3 minutes at 72° C, and the final extension at 72° C for 10 minutes (Hezayen *et al.*, 2002). The PCR product quality was verified on 1.0 % agarose gels and staining with ethidium bromide.

V. *Int*-PCR and gel electrophoresis:

The conserved regions for the integrase genes were amplified using the following reagents for a final volume of 12.5 µl : 1X Buffer, 3 mM Mg, 1X BSA New England®, 1.0 mM dNTP's, 10 pMol of degenerate primers hep35 and hep 36 (Table 2.1), 0.0104 U *Taq* polymerase (Promega) and 1 µl of template (15 ng/ µl).

Amplification cycles were the following: initial denaturation at 94 ºC by 5 minutes; 35 cycles of denaturation at 95 ºC; annealing at 52 ºC and extension at 72 ºC; the final extension was at 72 ºC for 7 minutes. The PCR product quality was verified in 1.5 % agarose gels stained with ethidium bromide.

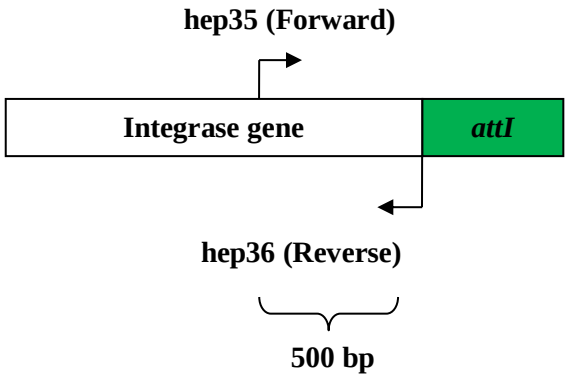


Figure 1.7 Diagram which demonstrates the common region for the integron-encoded integrase that was amplified in the PCR assay.

VI. Ligation and transformation:

The PCR product was purified using Wizard SV Gel and PCR clean up system (Promega, Madison, WI, USA) according to manufactures instructions and was ligated into pGEM Vector Systems (Promega, Madison, WI, USA). The ligation mixture was transformed into JM109 *Escherichia coli* by heat shock according to manufacturer's instructions.

VII. Alkaline –SDS Plasmid Minipreps and sequencing

Clones containing inserts were incubated overnight at 37ºC (1 ml). The plasmid isolation was performed using the Hanahan procedure. Basically, cells were resuspended in lysis buffer (25 mM Tris-HCl pH 8.0, 50 mM Glucose and 10 mM EDTA) followed by denaturalization with 0.2 N NaOH and 1% SDS solution of 5 M Potassium Acetate. The isolated plasmids were re-suspended in 50 µl of TAE and treated with RNAse (20 µg ⁄ µl). The plasmid was resolved by electrophoresis in agarose (1.5%) and visualized after staining with ethidium bromide (10ug/mL). Plasmid concentration was determined using the nanodrop.

VIII. DNA sequencing:

Fifty clones were randomly chosen and sent for sequencing at the UPR-Río Piedras Sequencing Facility and UW-htseq Sequencing Facility (Washington University at St. Louis, MO) using the T7 primer. Samples were prepared according to facility specifications.

IX. Characterization and Phylogenetic analyses of intI sequences:

The environmental integron integrases were edited in Bioedit and aligned against sequences in GenBank databases using ClustaX and Bioedit. The sequences from Int1, Int2, Int3, Int4, XerC, and XerD (GenBank accession number AAQ16665.1, AAT72891.1, AA2A3VO32355.1, 99031763 P0A8P6.1, and P0A8P8.1) were used as references for the identification of functional residues and domains. The tyrosine recombinase: XerC and Xer D were chosen to identify additional domains characteristic of integron integrases. Comparative amino acid sequence analyses were performed by constructing consensus neighbor-joining dendrograms using Mega4 software package as previously described (Rodríguez−Minguela et al., 2009). The dendograms were generated with p-distance model and tested by 2,000 boostrap replications.

Results:

Metagenomic DNA extractions from environments with moderate anthropogenic impacts from Puerto Rico:

Culebrones Cave soil, Solar Saltern Ponds and Coamo Thermal Springs samples were used for total metagenomic DNA. Figure 1.8 demonstrates the quality of ten metagenomic DNA extractions performed from Solar Saltern Ponds, Culebrones Cave, and Coamo Thermal Springs. Metagenomic DNA extracted using membrane and bead beading technique was quantified using the Nanodrop ND-1000 Spectrophotometer© and adjusted so there were 15 and 12ng of template for 16S rDNA region and integrase integron PCR amplification, respectively. Universal primers were used for the partial amplification of integron integrases (491bp) and 16S rDNA genes (900bp).

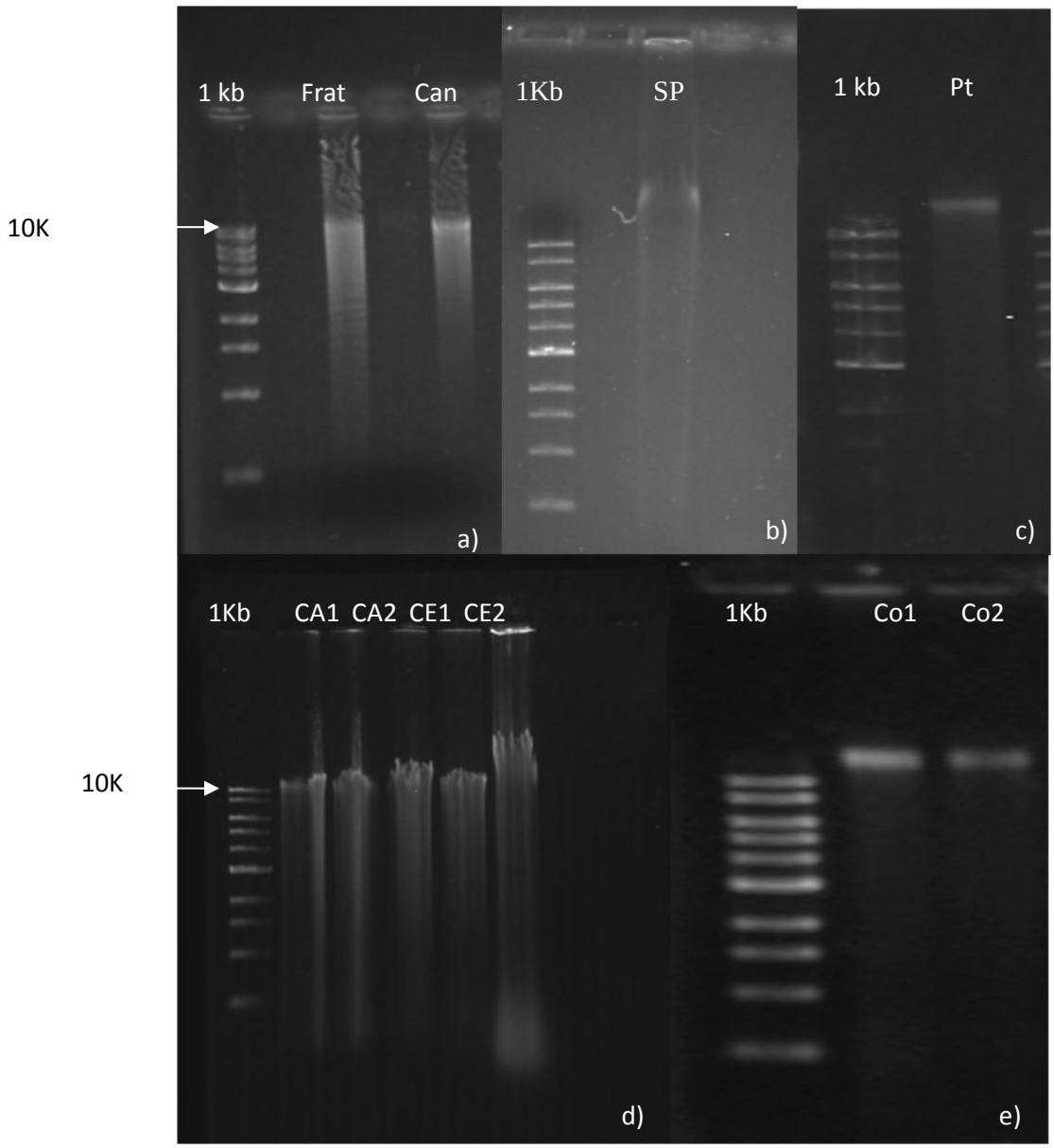


Figure 1.8 Figure 1.8 Metagenomic DNA extractions from the Solar Saltern Ponds (a,b,c), Frat =(Fraternidad), Can = (Candelaria), SP =(Salterns Ponds), Pt =(Puente), (d) soil from Culebrones Cave, CA1 and CA2= (soil within the cave), CE1 and CE2 = (soil at the cave entrance), and the Coamo Thermal Springs (e), Co1 and Co2 = (Coamo 1 and 2).

Metagenomic 16S rDNA from environments with moderate anthropogenic impact from Puerto Rico:

The quality of these metagenomic DNA´s were determined and then used as templates for 16S rDNA amplification by PCR. PCR amplicons were of approximately of 900 pb and the DNA markers were 1kb (Figure 1.11).

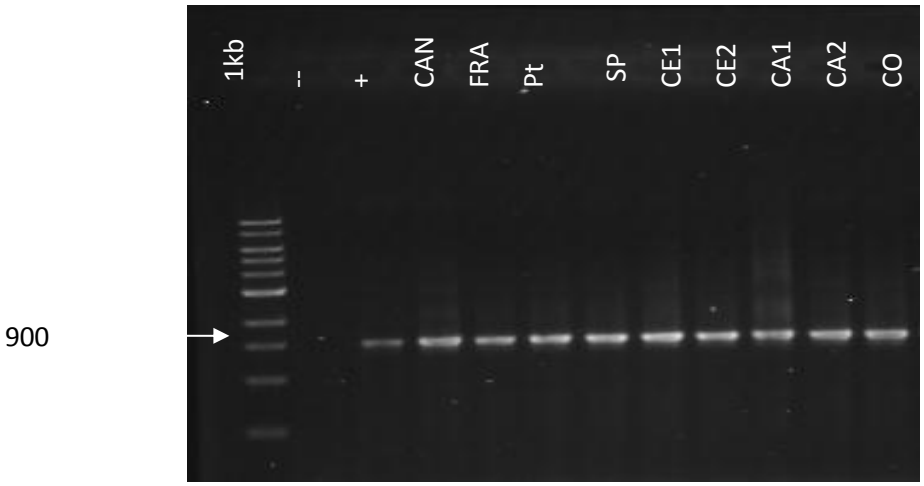


Figure 1.9 16S rDNA amplification from Metagenomic DNA using the universal primers Bact 1392R and BACT 27 from the Solar Saltern Ponds, Cueva Culebrones and the Coamo Thermal Springs. The positive control (+) is an 16S rDNA amplicon from a strain isolated from mangrove soil, and the negative control (–) is a PCR sample without template DNA. Frat =(Fraternidad), Can = (Candelaria), SP =(Salterns Ponds), Pt =(Puente), CA1 and CA2= (soil within the cave), CE1 and CE2 = (soil at the cave entrance), and Co1 and Co2 = (Coamo 1 and 2).

***Int*-PCR from metagenomic DNA from environments with moderate anthropogenic impacts from Puerto Rico:**

The metagenomic DNA's extracted were used as a templates for integrase gene amplification using degenerate primers hep35 and hep 36 nucleotide sequences complementary to the Box I and Box II domains found in integron integrases. The amplification was performed in triplicate and PCR products of approximately of 491 pb were obtained from of Solar Saltern Ponds, Culebrones Cave, and Coamo Thermal Springs (Figure 1.10 and 1.11). These were cloned for each place. It was necessary to perform optimization of the PCR annealing temperature in order to achieve optimal amplification of the desire products. The purified PCR products of each place

was cloned and nine clone libraries were constructed and 100 clones were obtained. Only 50 clones from each clone libraries were selected randomly for sequencing purposes.

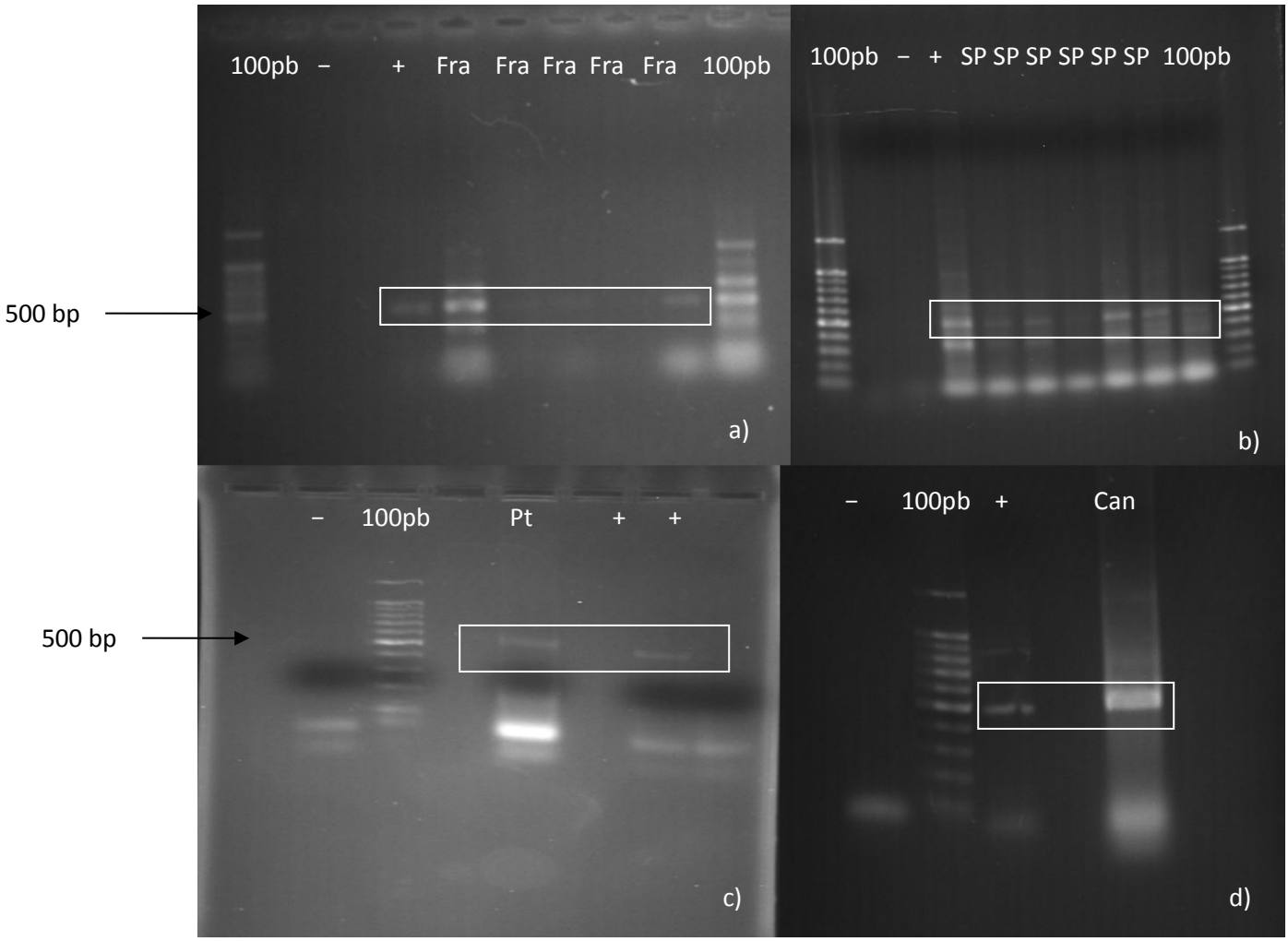


Figure 1.10 *Int*- PCR amplicons using universal, Hep35 and Hep36 of the total community DNA extracted from Fraternidad Lagoons as template. Positive control (+) contained integrase genes as template from Mona Island soil, and negative control (–) is a PCR sample without template DNA. Frat =(Fraternidad), SP =(Saltern Ponds), Pt =(Puente), Can = (Candelaria).

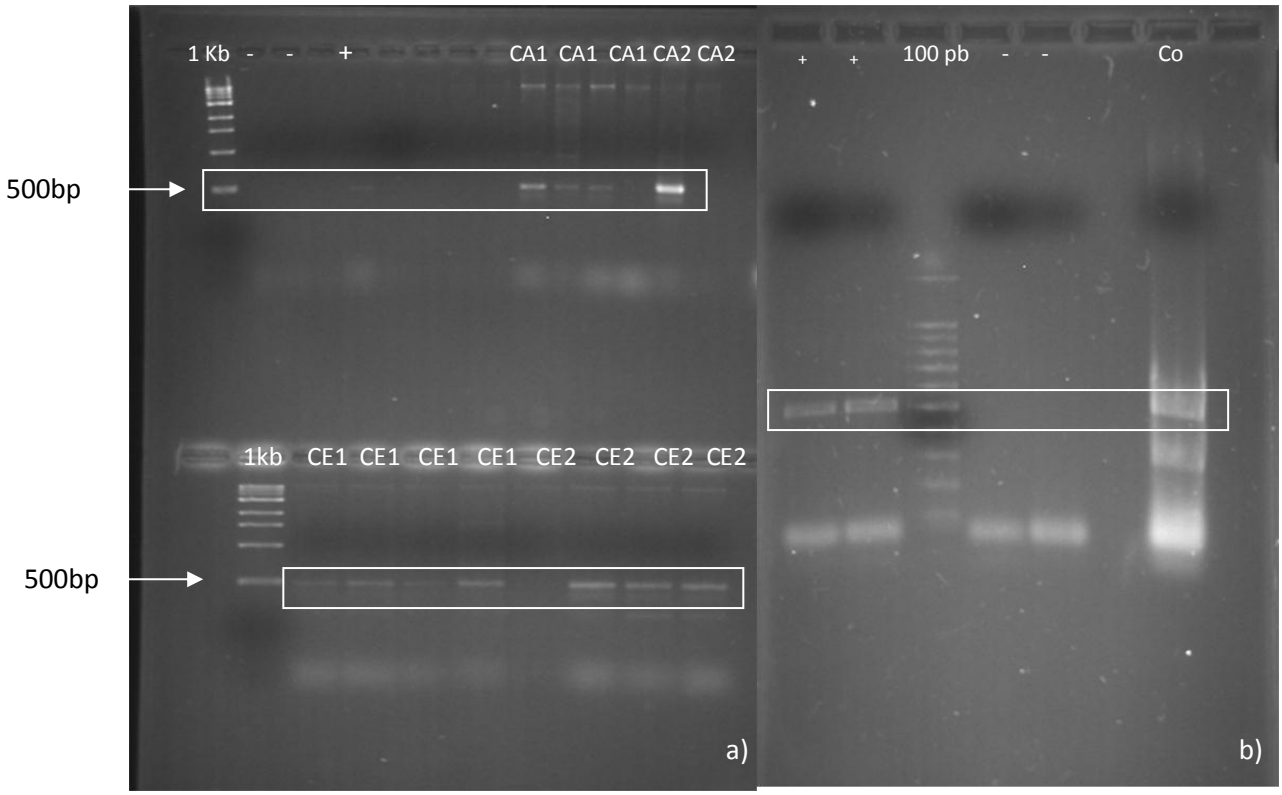


Figure 1.11. *Int*- PCR amplicons using universal, Hep35 and Hep36 primer and total community DNA extracted from Cueva Culebrones (a) CA1 and CA2= (soil within the cave), CE1 and CE2 = (soil of the entrance cave) and Coamo Thermal Springs (b) Co = (Coamo). Positive control (+) contained integrase genes as template from Mona Island soil, and negative control (–) is a PCR sample without template DNA.

Int Alignment analysis from environments with moderate anthropogenic impacts from Puerto Rico:

Although, the full length of IntI putative sequences were not retrieved by the restriction primer pair used, the integron integrase conserve motifs and residues critical for binding and recombination mechanisms could be indicative that recovery Int sequences can be functional. Environmental IntI sequences recovered were aligned against functional integron integrases of a class1 integrase described in *Escherichia coli*, IntI2 described in *Shigella sonnei* and IntI3 described in *Klebsiella pneumonia* (GenBank accession numbers: AAQ166651, AAT728911 and AAO323551, respectively). The numbers in the frames of the alignment were determined using Int4 (99031763) numbering because their crystal structure and recombination site were deter-

mined (MacDonald et al., 2006). Residue R143 corresponds to an arginine residue found within the Box I region of Int4.

Functional motifs which are characteristic of the tyrosine recombinase family such as: Patch (II and III) and Box (I and II) were detected in all recovery sequences from extreme environments (Fig. 1.12, Fig.1.13, and Fig. 1.14). The signature tetrad RHRY characteristic of the tyrosine recombinase family was detected. Also, these residues are enclosed within the Box II motif and corresponded to positions: H268, R271 and Y303. H268 is implicated in the appropiate orientation and stabilization of the transition state during the integrative reaction (Messier and Roy, 2001). Y303 is the nucleophile responsible for cleavage and forms a covalent intermediate by esterification. Moreover, amino acids related to catalytic and DNA binding functions were 100% conserved: K160, (Box I), A205, L206, K209 and F223 (additional domain), and G293 (Box II). Residues A205 and L206 play a role in positioning and stabilization of the active site with respect to the attC site. Residue K160 (Patch II) is an important amino acid in maintaining an also important function in the cleavage activity (Rodríguez-Minguela, et al., 2009).

The P211 (additional domain) was conserved 100% in all IntI sequences from Culebrones Cave and Coamo Thermal Springs (Fig. 1.12 and Fig.1.14), while 80% of the sequences from Solar Saltern Ponds were conserved. Residue A205 (additional domain) was conserved 100% in samples from Culebrones Cave and Coamo Thermal Springs. However, 80% of the residue 205 was conserved from Solar Saltern Ponds (Fig. 1.13), with an exception of p57 and can 47, where substitutions were observed by another hydrophobic amino acid (leucine) and polar residue (serine). Residue P211 (additional domain) was conserved 100% in all IntI recovery sequences from Culebrones Cave and Coamo Thermal Springs. Meanwhile, 80% of the residue P211 in the se-

quences from Solar Saltern Ponds was conserved and substituted by acid residue lysine and hydrophobic amino acid glycine (Fig. 1.13).

The crystal structure of VchInt4 and the role residues important for attachment and stabilization of the synaptic complex have been recently described (MacDonal et al., 2006). The residue W219 associated with attachment and residues P232, H240, and H241 are conserved 80 and 100% across the IntI sequences from Culebrones Cave and conserved 100% in sequences recovery from the Solar Saltern Ponds and Coamo Thermal Springs (Fig. 1.12, Fig.1.13, and Fig. 1.14). Residues P232, H240 and H241 are implicated in stabilizing the complex. Residue K145 was conserved 100% in all environment sequences except in the case of Int4 residue Q145 which appeared to be exclusive of the *Vibrio* clade (Rodriguez-Minguela, 2009).

Residues D191 and P211 (additional domain) are conserved 100% in IntI sequences recovery from the Culebrones Cave and thermal springs and 90% conserved in IntI sequences from Solar Saltern Ponds. Two other residues, L202 and D231, (additional domain) 80 and 90% were conserved in all sequences.

Residues L291, L292, G293, and H294 were also 100% conserved in all sequences from Culebrones Cave and Coamo Thermal Springs and have been implicated in having a key role in the recombination mechanism (Messier and Roy, 2001).

The IntI sequences recovery from Culebrones Cave (Fig.1.12) contains residues as P224 and V222 and were identified within the Patch III region with 90% conservation. A high degree of sequence conservation was observed within the Patch II region in which residues G159, G162, R165, and L169 between 80% and 90% were conserved. In the Box II the residues: H272, F274, A275, T276, L278, L279, D284, I285, R286, V288, Q289, L292 G 293 H294, T299, T300, I302,

T304, H305, V306 were conserved 100% . The residues H277, G282, T287, and D296 were conserved 99%. In addition, the residues E290, L307 were conserved 80% within of the Box II.

Similarly the Int sequences in the residues from Solar Saltern Ponds were identified as P224 (Patch III) with conserved 80%. Other residues were found in the Patch II; G162, R165 were 100% conserved and L169 was conserved 80%. A high degree of sequence conservation was observed within the Box II region in which residues H272, F274, A275, T276, I285, L292, T299, T300, I302, T304, H 305, V306 were conserved 100% and L307 was conserved 99%. L279 and D284 were conserved 90% within the Box II and Q289 was conserved 99%, s34 was substituted by the hydrophobic amino acid glycine. R286 is 99% conserved in all sequences from Solar Saltern Ponds. G293 and H294 were found conserved 100% and L292 was conserved 99%; all are important amino acids related to the recombination mechanism (Messier, N and Roy, P.H., 2001). Residue H277 was conserved 80% and substitution by other polar and hydrophobic amino acid residue as arginine, glutamine and alanine were detected. G282 and L307 were conserved 99% within of the Box II.

Other residues found in Coamo Thermal Springs (Fig.1.14) were found within the Patch II: G159, G162, K163, R165, L169 that were conserved 100%. In the Box II conserved 100% and residues were identified residues across all the sequences: H272, S273, F274, A275, T276, H276, H277, L278, L279, G282, D284, R286, V288, Q289, T299, T300, I302, T304, H305, V306 and L307. Within Box II, residues: I285, T287, D296, E290 and L307 were conserved 99%.

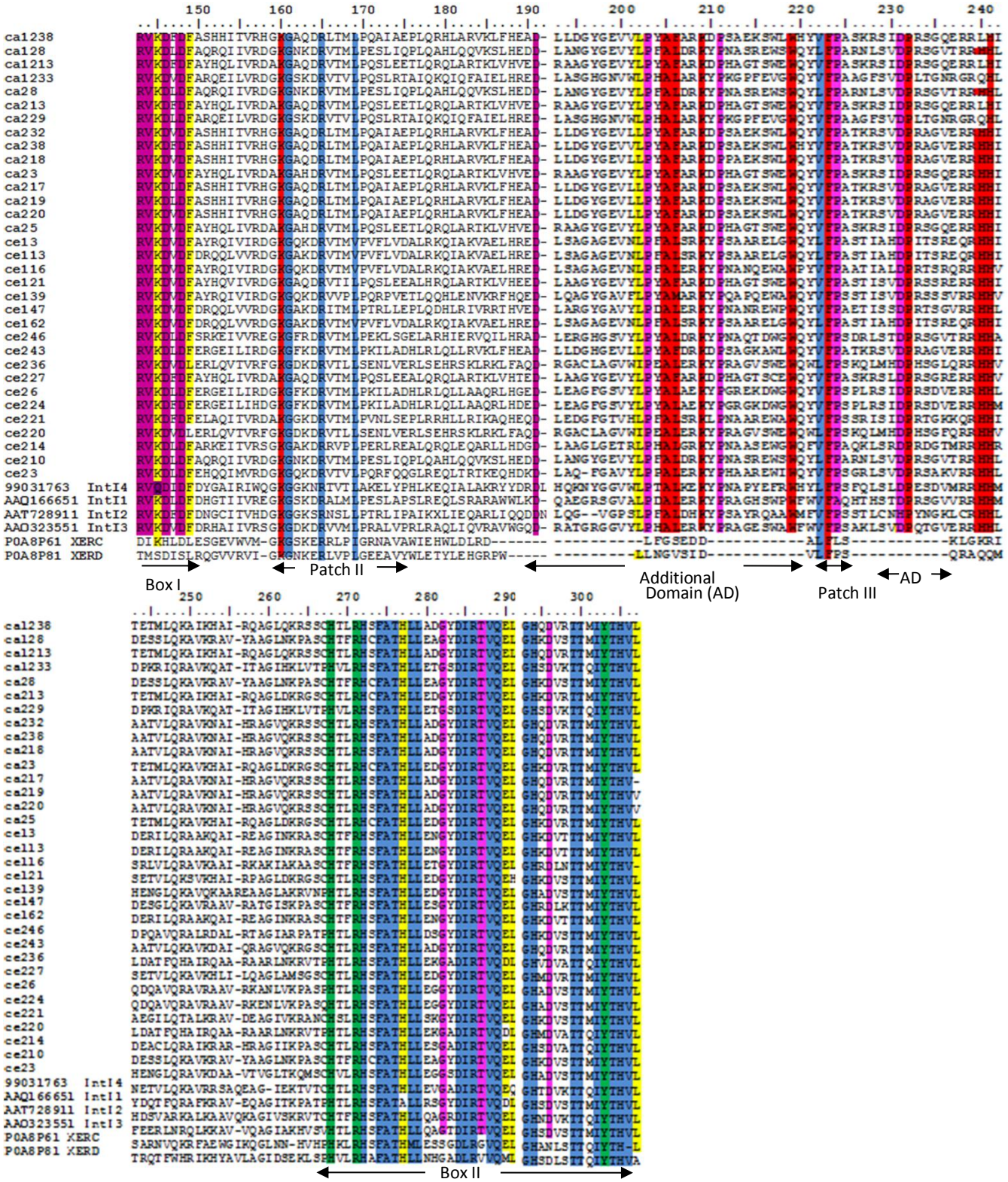
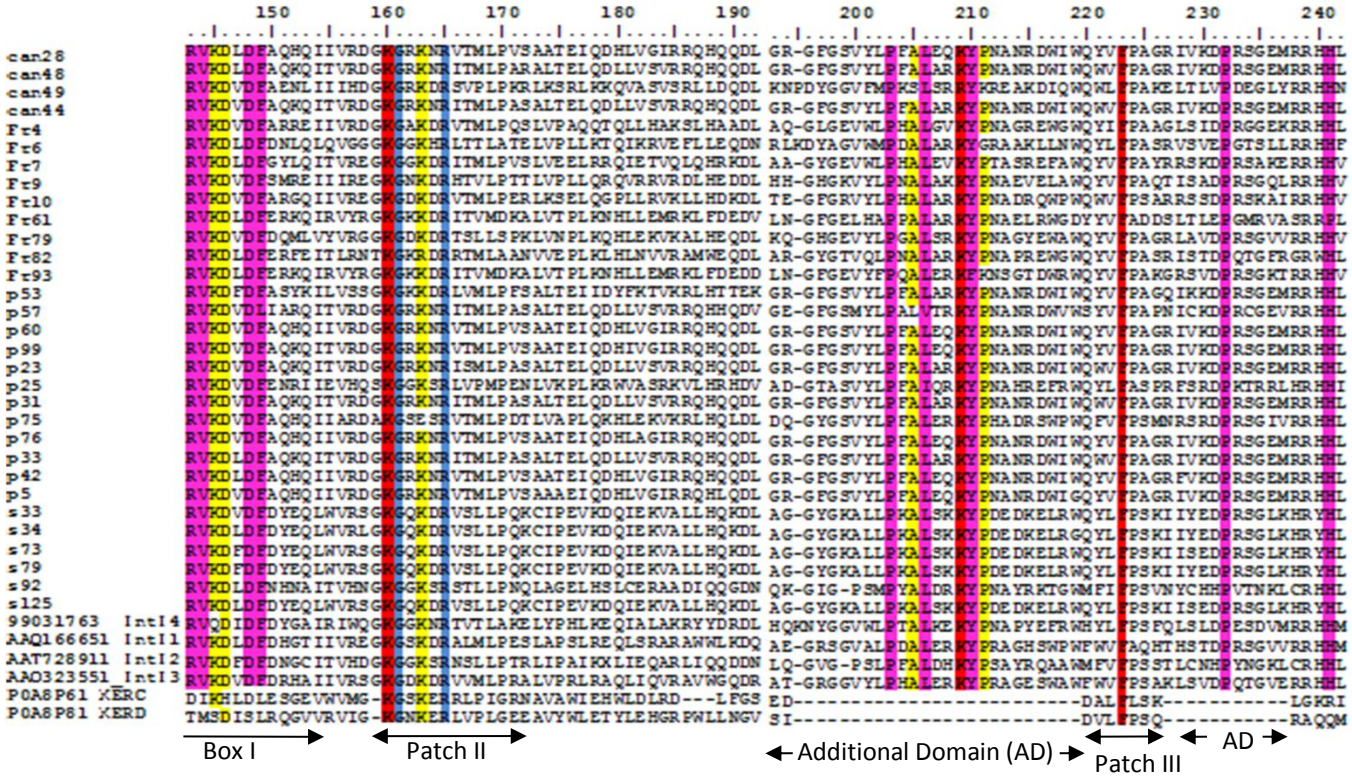
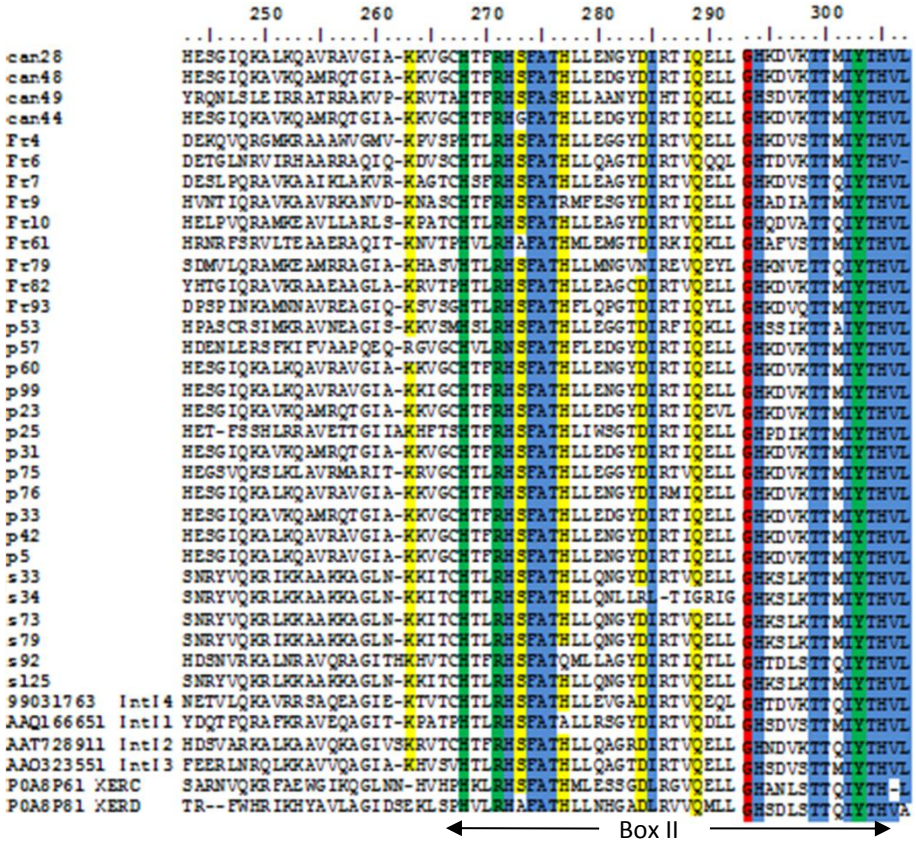

Box I
Patch II
Additional Domain (AD)
Patch III
AD
Box II

Figure 1.12 partial amino acid sequence alignment of environmental integrases from Culebrones Cave against other tyrosine recombinases, IntI class 1 from *Escherichia coli*, IntI 2 from *Shigella sonnei*, IntI 3 from *Klebsiella pneumoniae*, IntI 4 from *Vibrio cholerae* and tyrosine recombinases XerC and XerD from *E. coli*. The position of conserved motifs among integron-encoded integrases is indicated by segments labeled as Boxes I and II and Patches II and III and as additional domains (AD) under the alignment. The degree of conservation (percent identity) among equivalent residues is indicated as follows: blue (100%), yellow (95% and 85%). Conserved amino acids (100% identical) with functions related to protein folding, DNA binding, and recombination activity are highlighted in red, respectively. Green columns correspond to H, R, and Y residues from the conserved RHRY tetrad, which is characteristic of the entire tyrosine recombinase family **(**Nunes-Dubt, S., et al. 1998). GenBank accession numbers for the protein sequences of reference integrases (classes 1 to 4) and tyrosine recombinases XerC and XerD are AAQ16665, AAT72891, AAO32355, 99031763, P0A8P6, and P0A8P8, respectively.





FFigure 1.13 Partial amino acid sequence alignment of environmental integrases from the Solar Saltern Ponds against other tyrosine recombinases, IntI class 1 from *Escherichia coli*, IntI 2 from *Shigella sonnei*, IntI 3 from *Klebsiella pneumoniae*, IntI 4 from *Vibrio cholerae* and tyrosine recombinases XerC and XerD from *E. coli*. The position of conserved motifs among integron-encoded integrases is indicated by segments labeled as Boxes I and II and Patches II and III and as additional domains (AD) under the alignment. The degree of conservation (percent identity) among equivalent residues is indicated as follows: blue (100%), yellow (95% and 85%). Conserved amino acids (100% identical) with functions related to protein folding, DNA binding, and recombination activity are highlighted in red, respectively. Green columns correspond to H, R, and Y residues from the conserved RHRY tetrad, which is characteristic of the entire tyrosine recombinase family **(**Nunes-Du¨bt, S., et al. 1998). GenBank accession numbers for the protein sequences of reference integrases (classes 1 to 4) and tyrosine recombinases XerC and XerD are AAQ16665, AAT72891, AAO32355, 99031763, P0A8P6, and P0A8P8, respectively.

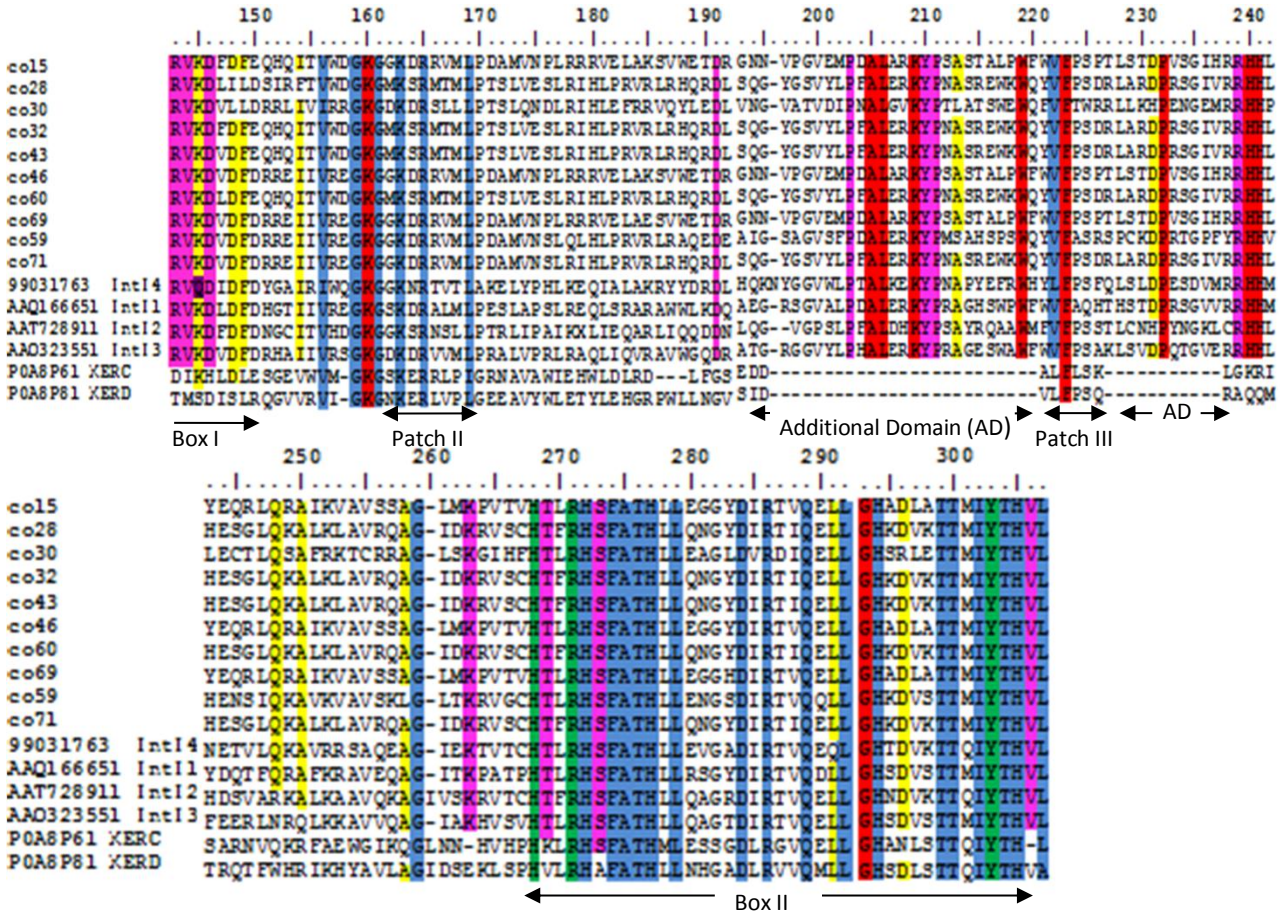


Figure 1.14 Partial amino acid sequence alignment of environmental integrases from the Coamo Thermal Springs against other tyrosine recombinases, IntI class 1 from *Escherichia coli*, IntI 2 from *Shigella sonnei*, IntI 3 from *Klebsiella pneumoniae*, IntI 4 from *Vibrio cholerae* and tyrosine recombinases XerC and XerD from *E. coli*. The position of conserved motifs among integron-encoded integrases is indicated by segments labeled as Boxes I and II and Patches II and III and as additional domains (AD) under the alignment. The degree of conservation (percent identity) among equivalent residues is indicated as follows: blue (100%), yellow (95% and 85%). Conserved amino acids (100% identical) with functions related to protein folding, DNA binding, and recombination activity are highlighted in red, respectively. Green columns correspond to H, R, and Y residues from the conserved RHRY tetrad, which is characteristic of the entire tyrosine recombinase family (Nunes-Dubt, S., et al. 1998). GenBank accession numbers for the protein sequences of reference integrases (classes 1 to 4) and tyrosine recombinases XerC and XerD are AAQ16665, AAT72891, AAO32355, 99031763, P0A8P6, and P0A8P8, respectively.

Phylogenetic analysis from integron integrases from environments with moderate anthropogenic impact from Puerto Rico:

Thirty integron integrases were found in soil from the two different places of Culebrones Cave in Arecibo. Additionally, twenty eight integron integrases were recovered in the screening of four places from the Solar Saltern Ponds in Cabo Rojo (Candelaria and Fraternidad Lagoon, ″puente″ and saltern ponds). Also, eight integron−encoded integrases were recovered from the Coamo thermal spring**.** The primers set used to amplify approximately 491bp of integron integrases were hep35 and hep36 complimentary to nucleotide sequences founded in the Box I and Box II domains of integron integrases (Table 2.1). All intI−like clones identified as integron integrases recovered from Culebrones Cave, the Solar Saltern Ponds, and the Coamo Thermal Spring created clusters in which the tyrosine recombinases represented by XerC and XerD (P0A8P6, and P0A8P8) were excluded. Most of the environmental integrases recovered from different environments with moderate anthropogenic impacts were different from the other previously described. The sequences corresponding to the clone s92 were the only genotype related to the integrase class2 (AAT28911) described in pathogenic strain *Shigella sonneii* (AAT728911) (Figure 1.15, cluster I, 66% identity). Clinical integrases class1 (AAQ166651) and 3 (AAO323551) formed one cluster where the most similar clones were from Coamo Thermal Springs (Figure 1.15, cluster 10) with 46 and 98% identity respectively. Related to IntI4 (99031763) is the clone Fr6 (Figure 1.15, cluster II, 55% identity) which was collected from the Cabo Rojo Solar Saltern Ponds.

In the dendogram constructed, one cluster exclusively consisted of sequences recovered from ″Puente″ and Candelaria (Figure 1.15, cluster 1, 63%-88% identity) and very distant from the clinical integron integrases. However, the sequences are similar to the integrase of uncultured

bacterium (ABD625711) whose origin is Mona Island's dry forest soil. Nevertheless, discrete cluster are observed from solar ponds (Figure 1.15, cluster 5, 98%-63% identity) related to integron integrase of manure soil origin, while sequences recovered from Fraternidad are disperse without any cluster formed. Discrete cluster consisting of sequences from Coamo Thermal Springs were detected (Figure 1.15). One cluster is observed to be related to integron class 1 and class 3, meanwhile other clusters are distant from the clinical integron integrases (Figure 1.15, clusters 10 and 2). Culebrones Cave sequences showed different discrete clusters (Figure 1.15, clusters 3, 4, 6 and 7) whose sequences are distant to the clinical integron integrases with identity percentage of 98% up to 66% and all related to integron integrase of uncultured bacterium from dry forest soil from Mona Island and peat soil.

The following sequences recovered from the Culebrones Cave and the Solar Saltern Ponds are nearest to an Int of cultured bacteria. Clone ce139 was related to integron integrases described as a representative of the reductive sulfur cycle, *Desulfurivibrio alkaliphilus* (YP00336917161) having a 71% *IntI* gene sequence similarity (Figure 1.15, cluster 4). Can49 related to Int described in *Verrucomicrobiae bacterium* (ZPO50599471) having 53% *IntI* gene sequence similarity (Figure 1.17). Sequences recovered from Culebrones Cave and Fraternidad Laggon were related to integron integrase of Gram negative and the rod shape opportunistic pathogen, *Pseudomonas stutzeri (*ACB468491), with identity percentage from 59 and 66% (Fig. 1.15, cluster 9).

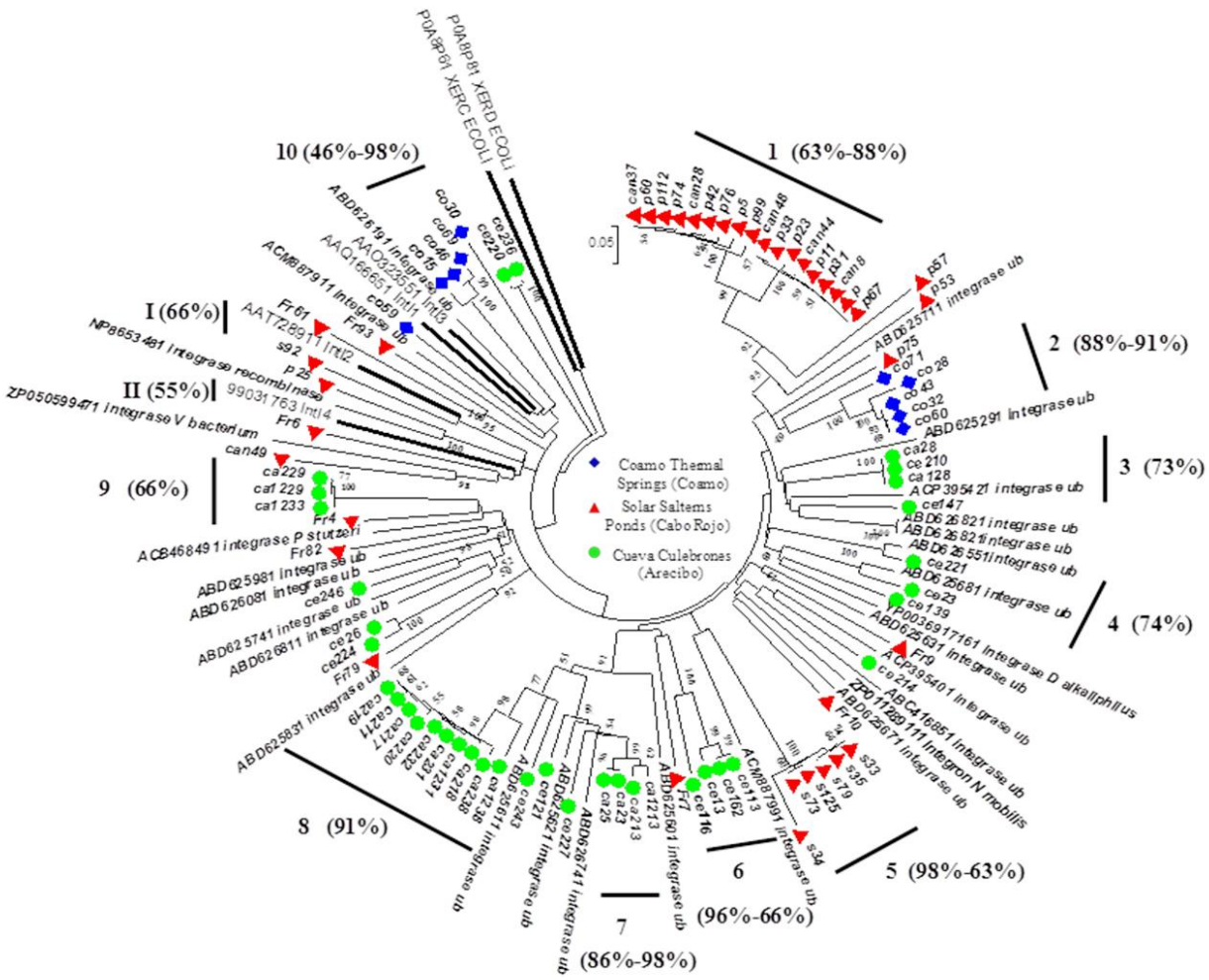


Figure 1.15 Consensus neighbor-joining tree illustrating the phylogenetic relationship of IntI sequences recovered from different extreme environments in Puerto Rico. Values represent the percentage of 1,000 boostrap replications that supported the branch. Outgroup references were designate with a thicker black branches highlight XerC, XerD, class IntI 1, IntI 2, IntI 3, and IntI4. Red triangle are sequences recovery from Solar Saltern Ponds, blue triangle circle from Coamo Thermal Springs and green circle are recovered sequences from Cueva Culebrones. Numbers 1 to 10 denote the clusters formed by recovery sequences and the percent identities of IntI sequences associated are shown in parentheses. Roman numbers (I and II) are recovery sequences related to clinical integron. Values of boostrap support below 49% are not demonstrated and Genbank accession numbers follow the outgroup sequences.

Discussion

It is important to gain information about the negative and positive effects of horizontal gene transfer and understating the role of the integron in these environments. This study represents the first attempt to determine the distribution and presence of the integrase of integron from environments with moderate anthropogenic impact and extreme conditions in Puerto Rico and the Caribbean. Validation of DNA quality was performed for all environmental samples, using specific primers for amplified the 16S rDNA. Optimizations were performed during IntI PCR, where annealing temperature range were 50-59º. We selected 52ºC as the annealing where an intense band was obtained and with less accessory bands. In previous work the annealing of 52º was selected to chose environment integron integrases not related to clinical settings (Rodríguez-Minguela et al., 2009); on the contrary, high annealing did not generate specific bands as the annealing of 56º, instead bands associated to integron integrases class 1 were generated. Although, the degenerate primers used amplify approximately 491bp of integrase integron genes, they were constructed using sequences of IntI class 1, 2 and 3 (White et al., 2000), which allowed amplified environments integron integrase not associated to clinical settings. Meanwhile the primers used by Nield et al., (2001) were primers which allowed to amplify integron platforms directly from the environment.

The molecular screening of integron integrases from environments with moderate anthropogenic impact in Puerto Rico demonstrate 68 novel integron integrases (thirty from Culebrones Cave, twenty eight from Solar Saltern Ponds and eight from Coamo Thermal Springs). The recovery of integron integrases from environments with moderate anthropogenic impact demonstrates that this mechanism not is restricted to clinical environments. The integron integrases found shared domain and motifs features of integron integrases. Moreover, the high conservation of important motifs related to recombination mechanisms, stabilization, and binding activity

show those probably are functional integron integrases. The low identities value (Fig.1.15) found in the recovery integron integrases with other IntI previously described indicate the diversity of integron integrase.

All sequences were aligned with previously described tyrosine integrases and functional integron integrases. The comparative phylogy and alignment performed demonstrated the integron integrase exclude other members of the tyrosine recombinases family, as are the XerD and XerC with a range of identity between 44−66%. XerD and XerC were used as reference for the identification of additional domain regions as principal feature of the integron integrases. Nevertheless, making inferences about the origins of environmental sequences recovered and their relationship with Xer are difficult because their evolution and origins are poorly understood. Clusters were observed for each environment when a consensus tree was performed for all environmental sequences. Sequences similar to integrase class 1, 2, and 3 were rare with exception Coamo Thermal Springs where these are closer to this class of integron integrase (46 and 98% identity).

The most distant to clinical integron integrase were found in majority in the Culebrones Cave, puente and salterns pond (Solar Saltern Pond) int−like. Fraternidad clones were very different and not forming any clusters within them. Only one int−like clone from Fraternidad Fr6 was the most closely related to IntI4 with 55% identity. Little sequences were closely related to integrase integron from cultured bacterium, an important reason to continue understanding the importance of the culture dependent technique at the moment of performing an analysis. Nine environment recovery sequences were related to culture bacterium: ce23 and ce139 related to *Desulfurivibrio alkaliphilus* (YP00336917161), ce214 and Fr10 related to *Nitrococcus mobilis* (ZP011289111); ca1233, ca1229 and ca229 to integrase of Gram negative and rod shape oppor-

tunistic pathogen, *Pseudomonas stutzeri (*ACB468491) and s92 to integron integrase class 2 from *Shigella sonnei.* and can49 related to Int described in *Verrucomicrobiae bacterium* (ZPO50599471).

In this study, thirty integron integrases were discovered were of in soil from four different places in the Culebrones Cave in Arecibo; twenty eight from Solar Saltern Ponds in Cabo Rojo (Candelaria and Fraternidad Lagoon, Puente and Saltern Ponds) and eight from molecular screening performed in Coamo Thermal Springs**.** Nemergut, D.R., et al. 2004 and Nield et al. 2001. Used a similar molecular approach and identified 14 and 3 new integrase genes from diverse environments.

The present work demonstrates that integron integrases not is restricted to clinical setting; in addition, the integron integrases may be a common feature of bacterial from caves, halophilic and thermophilic environments. Besides, the presence of novel intI genes implies the occurence of uncharacterized integrons and strengthens the importance of exploring these environments. In view of high diversity of integron integrase from different environment samples, it can create an extensive scenario of genetic functions where the prokaryotic cell can adapt quickly to environmental changes.

Part II. Sampling Procedure, Isolation, Characterization and *Int*-PCR of Isolates

Strains

I. Sample Processing and Isolation of Halophilic Bacteria:

At the Solar Saltern Ponds, eight samples containing 50 ml of saline water were collected (in duplicate) from the four different sampling points (Fraternidad and Candelaria Lagoons, Puente and solar ponds). The sampling was performed using Whirl Pack® bags and then samples were processed using the membrane filter technique. Saline water samples were homogenized with a pipette following the filtration of a volume of 25 ml in 47 mm, 0.45 μm (pore size) cellulose nitrate membrane filters (Whatman, Germany). Filtered membranes were inoculated into Luria-Bertani (LB) with different NaCl concentrations (0, 1, 5, and 8 %) at pH 7.5 and adjusted with 1M NaOH. All plates were incubated at 37°C. After three days of incubation colonies were selected and purified by the quadrant streak.

II. Alkalithermophilic bacteria culture media:

The *Thermus* medium (TM) was used for alkalithermophilic bacteria reactivation. TM consists of (in g/L) 8.0 peptone; 4.0 yeast extract and 2.0 NaCl, pH to 7.5 adjusted with 1M NaOH. The strains were incubated at 70º C.

III. Morphological and cultural characterization of halophilic strains:

Gram stains were performed using both heat-fixed smears and smears fixed in acetic acid 5% (Dussault, 1955). Macroscopic characteristics were documented using the classical characterization of colony appearance. Electron microscopy procedures were performed as previously described (Díaz-Muñoz and Montalvo-Rodríguez, 2005). The samples were examined using JEOL JSM-541 OL SEM microscope at 15 kv.

IV. Cryopreservation of halophilic strains:

Isolated strains were grown in 1.5 mL microtubes containing 930 µL of Luria Bertani at different NaCl concentrations (5 and 8 % w/v) and when cells were at logarithmic phase, 30 µL Dimethyl Sulfoxide (DMSO) were added and the sample was quickly frozen at - 80ºC. The culture was stored at - 80ºC.

V. DNA extraction

Isolated strains were grown in Luria Bertani at different NaCl concentrations (1, 5 and 8 % w/v) and incubated at 37 ºC. DNA was extracted using the bead beating technique suspended in lysis buffer (40 mM Tris-Acetate pH 7.8-8.0, 20 mM Sodium acetate pH 8.0, 1.0 mM EDTA pH 8.0, and 1% SDS) with lysozyme followed by chloroform extraction and ethanol precipitation. The DNA quality was verified on 0.8% agarose gels and later stained with ethidium bromide. All genomic DNA were used for PCR amplification.

VI. PCR amplification of 16S rDNA and gel electrophoresis:

Amplification of the gene encoding the 16S rRNA was perfomed by PCR using the forward primer Bact-27F and the reverse primer Bact-1392R (Table 2.1). The reaction mixture contained the following reagents at a final volume of 30 µl : 1X Buffer, 2.5 mM Mg, 1X BSA New England®, 2.5 mM dNTP's, 1 pMol of BACT 1392R and BACT 27F, 0.026 U *Taq* polymerase (Promega) and 1 µl of DNAtemplate (15 ng/ µl).

For 16S rRNA gene amplification, the PCR cycles consisted of initial denaturation for 5 minute at 94° C; 30 cycles of denaturation 1 minutes at 94° C, annealing 1 minute at 50 ° C and extension 3 minutes at 72° C, and final extension at 72° C for 10 minutes (Hezayen *et al.*, 2002). PCR amplicons were purified using the MinElute PCR purification kit (USA QIAGEN Inc.) according to manufacturer instructions and concentration determined with nanodrop.

VII. *Int*-PCR:

The PCR reaction was performed using the following reagents at a final volume 12.5 µl : 1X Buffer, 3 mM Mg, 1X BSA New England®, 1.0 mM dNTP's, 5 pMol of hep35 and hep 36, 0.0104 U *Taq* polymerase (Promega) and 1 µl of template (15 ng/ µl).

PCR cycles were the following: initial denaturation at 94 ºC by 5 minutes; 30 cycles of denaturation at 95 ºC, annealing of 52 ºC and extension of 72 ºC, the final extension is of 72 ºC by 7 minutes. The PCR product quality was verified on 1.5 % agarose gels and staining with ethidium bromide.

VIII. DNA sequencing:

Purified PCR products were sent for sequencing to UPR-Río Piedras Sequencing Facility and UW-htseq Sequencing Facility (Washington University at St. Louis, MO). Samples were prepared according to facility specifications.

IX. Sequence and Phylogenetic analyses:

Genomic integron integrases were edited in Bioedit and aligned against sequences in BLASTX using ClustaX. The sequences from Int1, Int2, Int3, XerC, and XerD (GenBank accession number AAQ16665.1, AAT72891.1, AA2A3VO32355.1, P0A8P6.1, and P0A8P8.1 were used as reference for identification of functional group and domains. Comparative amino acid sequences were performed constructing consensus neighbor-joining dendrograms using Mega 4 software package as previously described (Rodríguez−Minguela et al., 2009). The dendograms were generated with p-distance model and tested by 2,000 boostrap replications.

Table 2.1 Oligonucleotides used for PCR analyses.

Application	Primer	Sequence (5' to 3')	Product size (pb)	Annealing Temperature ºC	Reference
Amplification of 16S rDNA	Bact-27F 1392R	AGAGTTTGATCMTGGCTCAG ACG GGC GGT GTG TAC A	1365	50	Amman et al., 1995
Amplification of integron encoded integrases	hep35 (FWD)	TGC GGG TYA CCT ACA AAG CC	491	52	White et al., 2000
	hep36 (REV)	CAR CAD ATG CGT RTA RAT	491	52	
Promoter region of T7	T7	TAA TAC GAC TCA CTA TAG GG	variable	50	
Promotor region of SP6	SP6	ATT TAG GTG ACA CTA TAG AA	variable	50	

Results:

Morphological characterization

Samples from Solar Saltern Ponds, Cabo Rojo, showed growth on agar plates containing Luria Bertani medium with 5 and 8% (w/v) NaCl after a 3 days at 37ºC. A total of 25 strains were isolated from Candelaria and Fraternidad lagoons, ″puente″ and the salterns ponds. Two strains PII5 and SII5 were positive for integron encoded integrases. Morphological characterization was based on the classical macroscopic technique of colony appearance. Microscopical characterization was performed using the Gram staining procedure with acetic acid fixation as described by Dussault (1995). Strain PII5 is a Gram positive curved rod and grew in LB with 5% (w/v) NaCl (Fig. 2.1). Strain SII5 is a Gram negative rod and grew in LB with 8% (w/v) NaCl (Fig. 2.1). Both isolates showed colonies with entire and flat morphologies.

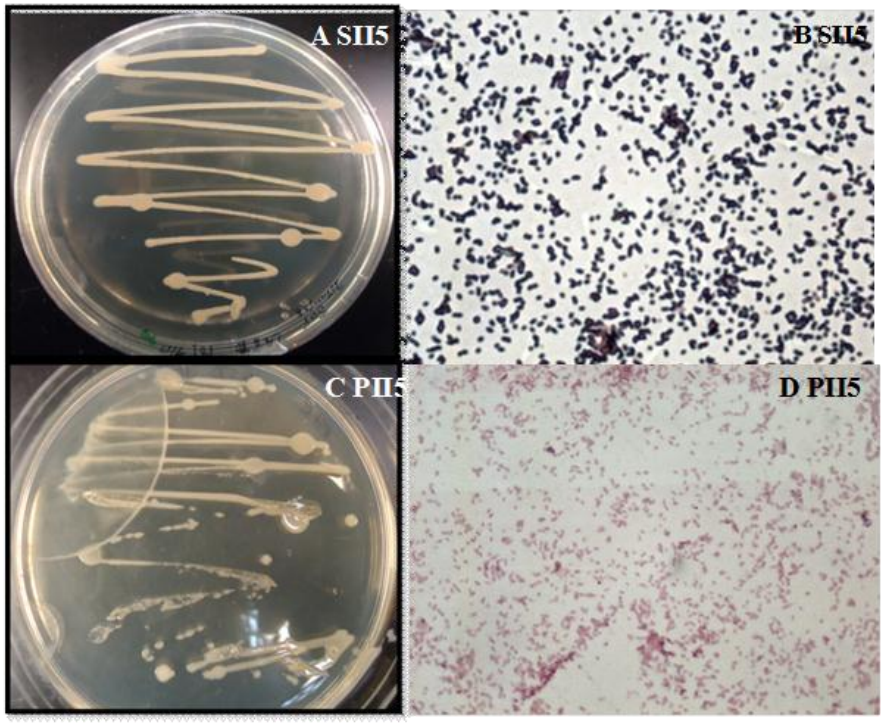


Figure 2.1 Macroscopical and microscopical characteristics of strain SII5 and PII5 isolated from the Solar Saltern Ponds on Luria Bertanie medium, curved rods strain using light microscope.

Strain CS91 was isolated in a previous study from the Thermophilic Springs, Coamo on TM medium (Burgos, A., 2007). Cells are gram positive long rods (Fig.2.1) with irregular and entire, flat, white colonies.

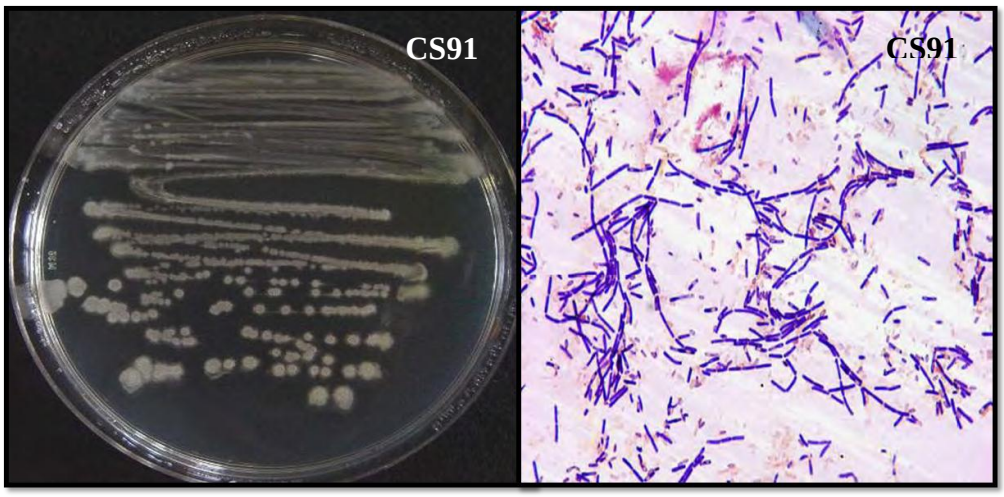


Figure 2.2 Macroscopical and microscopical characteristics of strain CS91 isolated in a previous study (Burgos, 2007) from the Thermophilic Springs, Coamo on TM, using light microscopy.

Genomic DNA extractions from prokaryotic strains isolated from Solar Saltern Ponds in Cabo Rojo and Coamo Thermal Springs.

Genomic DNA from strains isolated from the Solar Saltern Ponds and Coamo Thermal Springs was extracted using the organic extraction protocol with phenol/chloroform. Figure 2 shows the quality of genomic DNA extraction. Genomic DNA was quantified using the Nanodrop ND-1000 Spectrophotometer© and concentration was adjusted to 15ng of template for 16S rDNA and integrase integron PCR amplification.

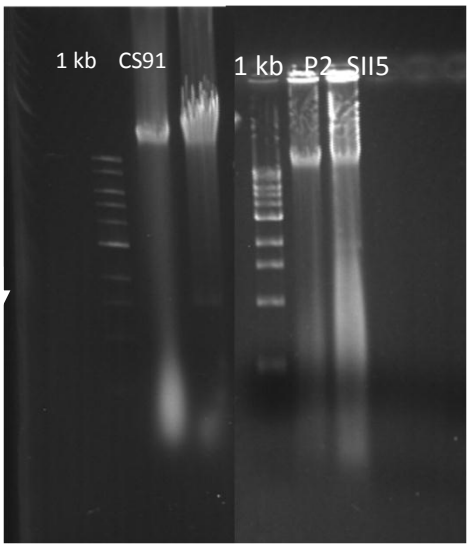


Figure 2.3 Genomic DNA extraction from prokaryotic cells isolated from the Solar Saltern Ponds PII2 and SII5 and CS91 isolated from the Coamo Thermal Springs by Burgos (2007).

Amplification of the 16S rDNA from strains from Solar Saltern Ponds and Coamo Thermal Springs:

The quality of the metagenomic DNA extraction was determined through 16S rDNA amplification by PCR. PCR amplicons were approximately 873 pb in size (Figure 1.11). It was necessary to perform annealing optimization of the PCR parameters for some strains in order to achieve optimal amplification. 1

873bp

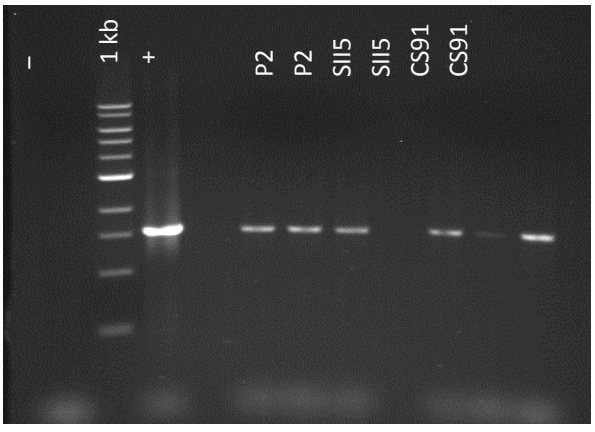


Figure 2.4 16S rDNA amplification using universal primers Bact-1392R and Bact 27-F of the isolated strains from the Solar Saltern Ponds.

***Int*-PCR from genomic DNA from strains isolated from Solar Saltern Ponds and Thermal Springs:**

A PCR product of 500 pb corresponding to a putative integrase was obtained by PCR from two prokaryotic strains isolated from Solar Saltern Ponds and one strain previously isolated from the Thermal Spring (Burgos, 2007). Amplicons were purified and sent for sequencing (Figure 2.4).

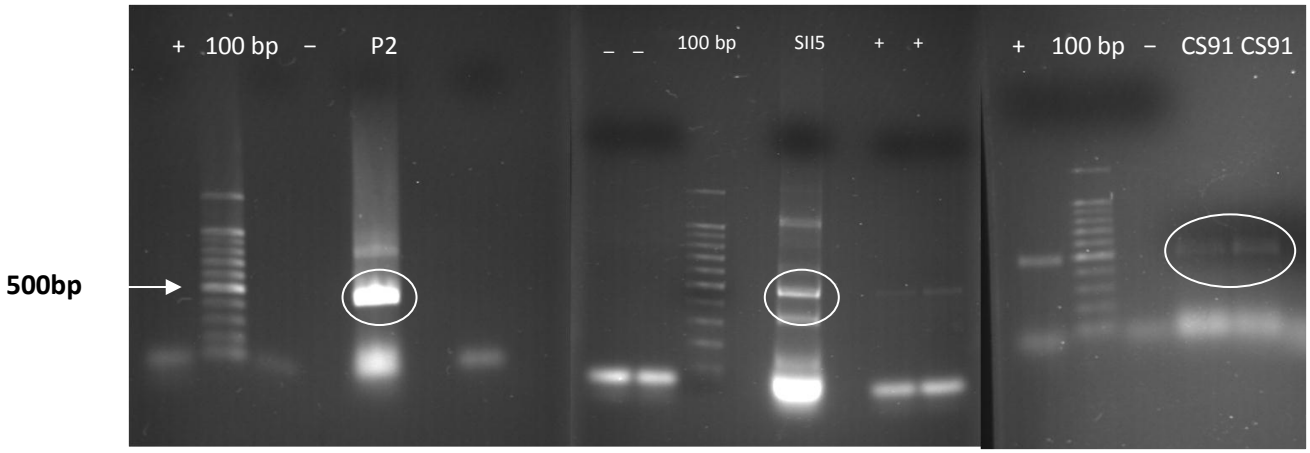


Figure 2.5 *Int*- PCR amplicons of PII5 strain isolated from the Solar Saltern of Cabo Rojo. Positive control contained integrase genes as template, and negative control without template DNA.

Integrase alignments analysis from strains PII5, SII5 and CS91:

Although the entire IntI putative sequences could not be retrieved using the primers hep35 and hep36 (Table 2.1), the integron integrase conserve motifs and residues critical for binding and recombination mechanism which could be indicative that recovered Int sequences can be functional. In addition, these sequences were compared against functional integron integrase in order to characterize the IntI sequences obtained from the halophilic and thermophilic strains. IntI sequences recovered from isolates were aligned against functional integron integrase of a class1 integrase described in *Escherichia coli*, IntI2 described in *Shigella sonnei* and IntI3 described in *Klebsiella pneumonia* (GenBank accession numbers: AAQ166651, AAT728911 and AAO323551, respectively). The numbers in the frames of the alignment were determined using

Int4 (99031763) numbering, because their crystal structure and recombination site were determined (MacDonald et al., 2006). Residue R143 correspond to an arginine residue found within the Box I region of Int4.

Important motifs which are characteristic of the tyrosine recombinase family, such as Patch (II and III) and Box (I and II), were detected. Residues of the conserved tetrad RHRY, characteristic of all tyrosine recombinase family, were detected in the following positions: H268, R271 and Y303, and each amino acid was located inside of the Box II motif. The principal roles of these residues, specifically H268, are appropriate orientation of the DNA and stabilization of transition state (Messier and Roy, 2001). However, Y303 is the nucleophile responsible for the cleavage; in addition it forms a covalent intermediate by esterification.

Important residues relationship with catalytic and DNA binding functions were identified with 100% conserved: K160 inside of Box I, A205, L206, K209 and F223 inside of additional domain, G293 inside of Box II. The residue K160 is an important amino acid found in Patch II and maintains a possible important function in the cleavage activity (Rodríguez-Minguela et al., 2009). The residues A205 and L206 play a role in positioning and stabilization of active site of attC. The P211 was 100% conserved in all IntI sequences inside an additional domain.

The crystal structure of VchInt4 was determined and important residues for attachment and stabilization of synaptic complex during recombination process were identified. Residue W219 was associated with attachment and residues P232, H240 and H24 were implicated in stabilizing the complex during recombination process and were 100% conserved. Residue, K145 is present in all genomic sequences recovered except in the case of Int4 where the presence of Q145 appeared to be exclusive of the *Vibrio* clade (Rodríguez-Minguela et al., 2009).

Residue D191 is 100% conserved across the IntI sequences from isolates. Residues, G159, K160, G161, K163, R165 within the Patch II region were 100% conserved. A high degree of sequences conservation was observed within the Box II region in which residues: R271,H272, S273, F274, A275, T276, G282, D284, I285, R286 G293, H294, T299, T300, I302, Y303 T304, H305, V306 and L307 were 100% conserved. Residues L291, L292, G293, and H294 were 99-100% conserved in all Int sequences from isolates and have been implicated in having a key role in the recombination mechanism (Messier and Roy, 2001).

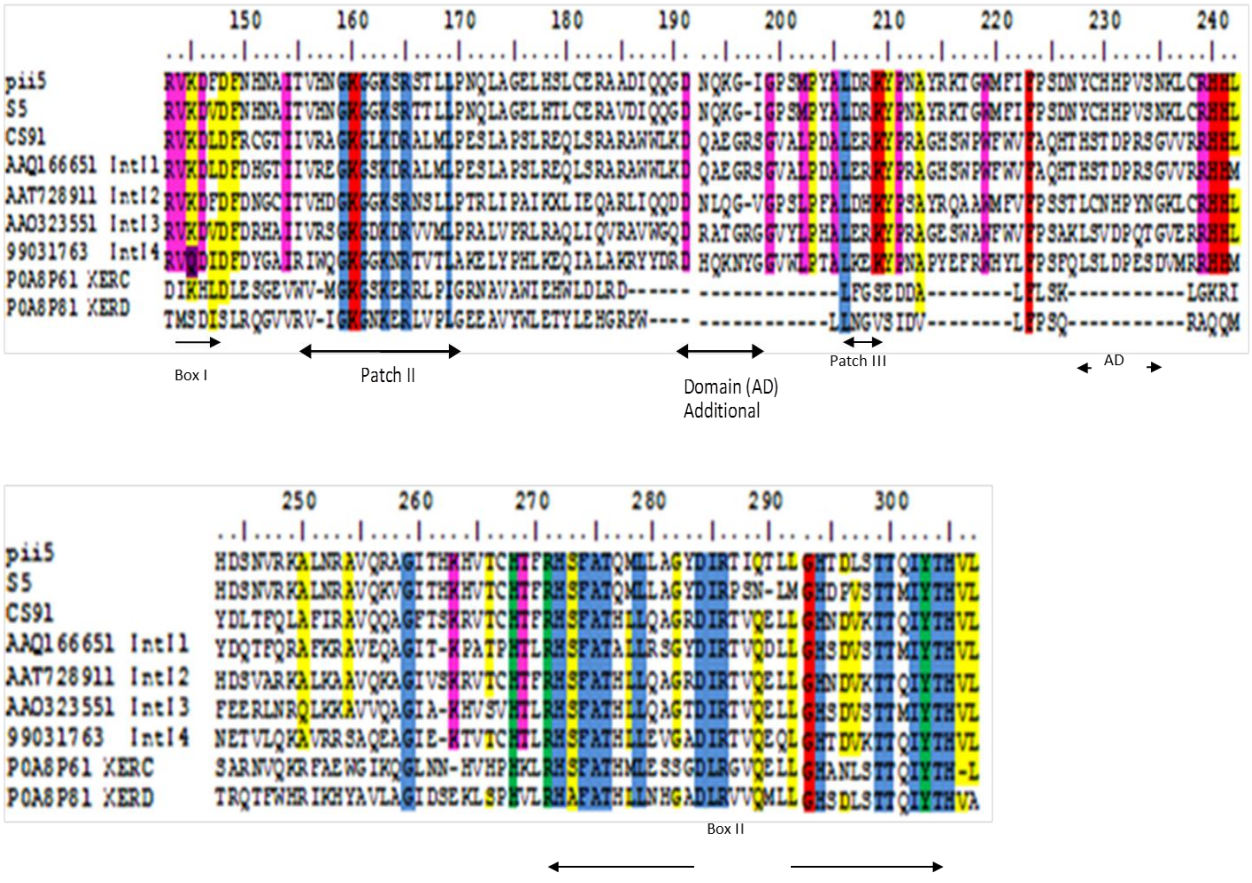


Figure 2.6 Partial amino acid sequence alignment of integrases from strains isolated against other tyrosine recombinase, IntI class 1 from *Escherichia coli*, IntI 2 from *Shigella sonnei*, IntI 3 from *Klebsiella pneumoniae*, IntI 4 from *Vibrio cholerae* and tyrosine recombinases XerC and XerD from *E. coli*. The position of conserved motifs among integron-encoded integrases is indicated by segments labeled as Boxes I and II and Patches II and III and as additional domains (AD) under the alignment. The degree of conservation (percent identity) among equivalent residues is indicated as follows: blue (100%), yellow (95%

and 85%). Conserved amino acids (100% identical) with functions related to protein folding, DNA binding, and recombination activity are highlighted in red, respectively. Green columns correspond to H, R, and Y residues from the conserved RHRY tetrad, which is characteristic of the entire tyrosine recombinase family (Nunes-Du¨bt, S., et al. 1998). GenBank accession numbers for the protein sequences of reference integrases (classes 1 to 4) and tyrosine recombinases XerC and XerD are AAQ16665, AAT72891, AAO32355, 99031763, P0A8P6, and P0A8P8, respectively.

Phylogenetic analysis of IntI from halophilic and thermophilic strains:

Three integron−encoded integrases were recovered from the isolated strains**.** These sequences were analyzed using multiple-sequence alignment made by using the Clustal W program and neighbor joining analysis with Mega 4 (Tamura et al., 2007). The sequences identified as integron integrases recovered from halophilic and thermophilic strains grouped clusters which excluded the tyrosine recombinases represented by XerC and XerD. Two clusters were formed with these sequences. The subsequent sequences formed one cluster; PII5 and SII5 the sequences obtained from two halophilic strains were closely related to IntI2 of an uncultured bacterium clone (ACM887541) and IntI2 from *Shigella sonnei*, with 64 and 68% identity respectively. The sequence CS91 found in thermophilic strain was closely related to IntI 1 described in *Escherichia coli* (AAQ166510) with 85% identity. Inside this cluster, Int3 from *Klebsiella pneumoniae* and IntI4 from *V. cholerae* formed their own independent branch (Fig. 2.7).

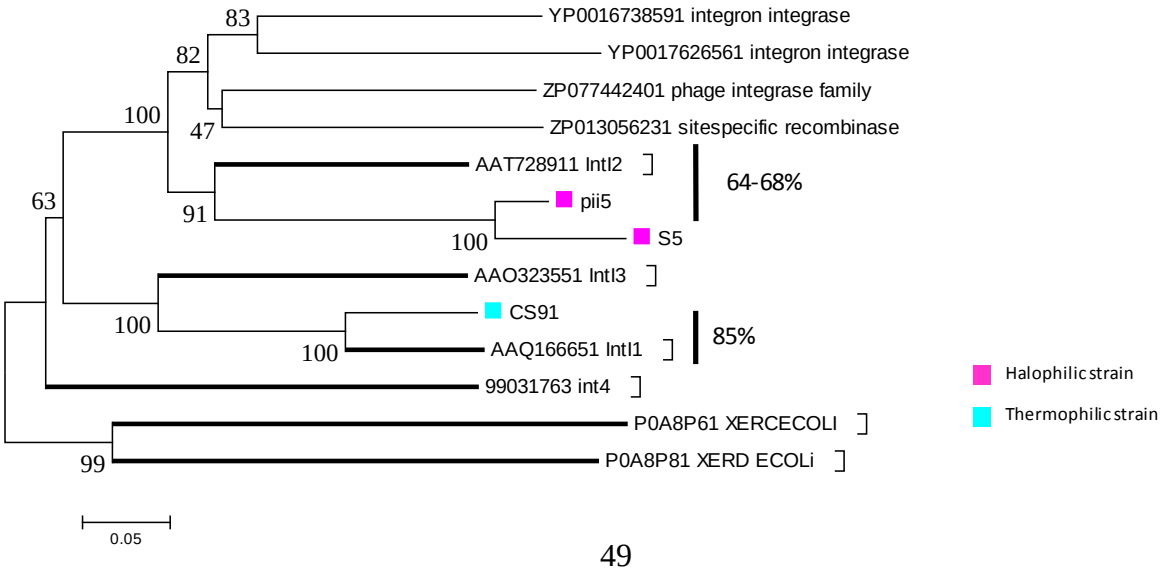


Figure 2.7 Consensus neighbor-joining tree (compared positions for 3 amino acid residues), illustrating phylogenetic relationship of IntI sequences recovery from halophilic and thermophilic strains. Values represent the percentage of 1,000 boostrap replications that supported the branch. Outgroup references were designate with a thicker black branches highlight (XerC, XerD, class IntI 1, IntI 2, IntI 3, and IntI4. Numbers select the clusters formed by recovery sequences and the percent identities of IntI sequences associated. Values of boostrap support below 20% are not demonstrated and Genbank accession numbers follow the outgroup sequences.

Phylogenetic analysis of 16S rDNA from halophilic and thermophilic strains:

The phylogenetic relationship of these sequences was determined using Clustal W program and neighbor joining analysis with Mega 4 (Tamura et al., 2007). Isolated strains from Solar Saltern Ponds were analyzed using the 16S rDNA gene and demonstrated that strain SII5 is closely related with the genus *Bacillus* and the closest relative was *Bacillus safensis* FO 036B^T with a boostrap value of 99%. Strain PII5 belonged to the *gamma-Proteobacteria* and is phylogenetically close to *Salinivibrio costicola* DSM 16359^T with boostrap value of 99% (Fig. 2.9).

Phylogenetic analysis using the 16S rDNA gene demonstrated that strain cs91 is closely related with to the genus *Geobacillus*, and the closest relative was *Geobacillus kaustrophilus* NCIMB 8547^T, with boostrap value of 95% (Fig. 2.10).

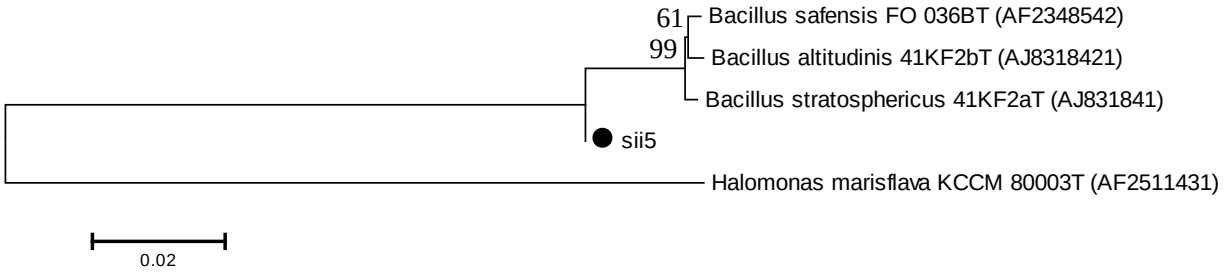


Figure 2.8 Neighbour-joining distance tree using 16S rDNA sequences of SII5 isolated from Solar saltern Ponds in Cabo Rojo and closely related species. Boostrap values higher than 50% are shown. *Halomonas marisflava* KCCM 80003T (AF2511431) was used as the outgroup.

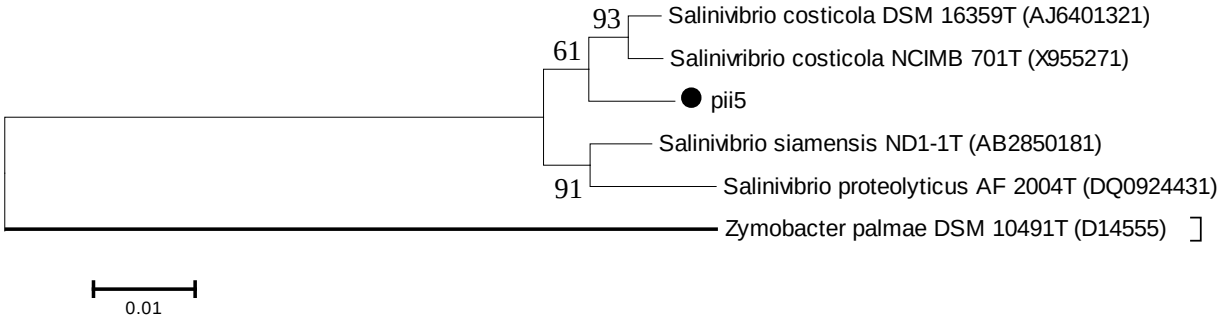


Figure 2.9 Neighbour-joining distance tree using 16S rDNA sequences of PII5 isolated from Solar Saltern Ponds in Cabo Rojo and closely related species. Boostrap values higher than 50% are shown. *Zymobacter palmae* DSM 10491T (D14555) was used as the outgroup.

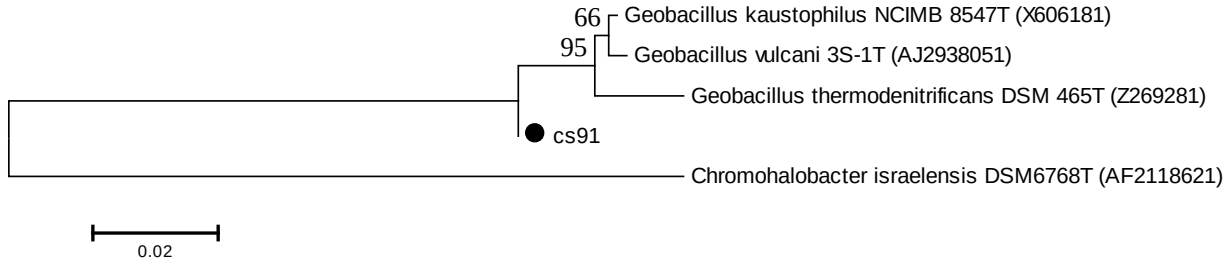


Figure 2.10 Neighbour-joining distance tree using 16S rDNA sequences of cs91 previously isolated by Burgos, A. from Coamo Thermal Springs and closely related species. Boostrap values higher than 50% are shown. *Chromohalobacter israelensis* DSM6768T (AF2118621) was used as the outgroup.

Discussion:

The objective of this part of the research was focused on the distribution and presence of integrases in strains isolated from environments with moderate anthropogenic impact and extreme conditions in Puerto Rico such as hypersaline water of the Solar Saltern Ponds in Cabo Rojo and the Coamo Thermal Springs. This study represents the first attempt to determine the presence of the integrase of integron into strains isolated from environments with moderate anthropogenic impact and extreme conditions in Puerto Rico and the Caribbean**.** Validation of DNA quality was performed for all strains, using 16S rDNA primers. Two bacterial isolates from Solar Saltern Ponds and one reactivated from one collection from Coamo Thermal Springs were screened for the presence of integron integrases by PCR. The genomic DNA of each bacterial was subject to 16S rDNA for phylogenetic analyze and determination of genus. SII5 a gram positive and rod shape bacterium isolated from Solar Saltern Ponds with 30% NaCl related phylogenetically to *Bacillus safensis*. *B. safensis* is a bacterium highly resistant to gamma and UV radiation and was the first isolated from spacecrafy-assembly facility of the Jet Propulsion Laboratory, Pasadena, California (Satomi et al., 2006). In addition, Gram-positive bacteria are major reservoirs of integron class 1 from aviar fecal material (Nandi et al., 2004). The strains SII5 and PII5 contain integrase related to integron integrase class 2 with domain and motifs feature of integron integrases, although the premature stop codon X179 was not.

Strain PII5 strain isolated hypersaline ponds with 8% NaCl was phylogenetic related to the genus *Salinivibrio* (Gamma-proteobacteria) which is gram negative curved rod shaped bacterium. Many bacterial isolated from sea water are found in hypersaline ponds as *Salinivibrio costicola subsp. vallismortis* isolated from a hypersaline pond located in Death Valley, California (Huang et al., 2000). The creation of this genus was a consequence of difference between *Vibrio*

The strain previously isolate from Coamo Thermal Springs is a gram positive rod− shaped bacterium close to genus *Geobacillus*. Their integrase integron is close to integron integrase class 1 with domain and motif features of integron integrases. The existence of high conservation of important motifs related with recombination mechanism, stabilization and binding activity show those probably are integron integrase functional. The genus of *Geobacillu*s provokes the industrial interest for their thermostable enzyme used in biotechnological processes. Previous work show integron integrase in thermophilic bacteria of the genus of *Sulfobacillus,* although this work amplification of possible cassette with specific primers was performed (Ghauri et al., 2003), integron integrases have been reported in other extreme bacteria as *Acidithiobacillus ferooxidans* (Rowe-Magnus and Mazel, 2001).

The present work demonstrates that integron integrases are not restricted to pathogenic and multidrug resistant bacteria, in addition the integron integrases may be a common feature of bacterial isolated from halophilic and thermophilic environments. Mechanism of horizontal gene transfer, as integron can take place in halophilic, thermophilic in and soil cave environments. This is the first attermpt to study the integron integrases from environments with moderate anthropogenic impact and extreme conditions in Puerto Rico and the Caribbean**.** Moreover the recovery of integron integrase from isolated areas is a great opportunity to advance investigation in biotechnology.

Conclusions

- Molecular screening from environments with moderade antropogenic impact and extreme conditions (Solar Saltern Ponds, Culebrones Cave and Coamo Thermal Springs) demostrated the presence of 66 novel integron encoded integrases with motifs features of integron integrases. This might suggest integron encoded intregrases can be found at non clinical related environments. The ecological role of these novel integrases is still unknown.
- Integron encoding integrases were found on the gram-positive isolates: SII5 related to *Bacillus* (99% similarity) and CS91 related to *Geobacillusis* (98% similarity). This provides additional evidence of the presence of this system in gram-positive bacteria.
- Isolate PII5 related to the family *Vibrionaceae* showed the presence of a novel integron encoded integrase this could be one of the first report inside of the genus *Salinivibr*io (99% similarity).

Recommendations

- This study demonstrated that integron integrases are distributed in environments with moderate anthropogenic impact from Puerto Rico, although the bacterial that carry these integrases are unknown. Important development techniques for detected the prokaryotic population associated to this integron integrases, were used without the need to perform culture technique dependent.
- The metagenomic technique is a useful technique to obtain complete and intact genes, without requiring PCR technique and primer design.
- Design different media that allow the isolation of different bacteria and determine preence or absence of integron integrases.
- Design a temporal study to compare prokaryotic diversity during different seasons of the year and associate it with the presence or absence of integron integrases.
- Determine the stability of integrons during a period of time in environments with modeate anthropogenic impact.
- Perform a gene cassette recovery and determine their functions using culture dependent and independent techniques.

Literatured Cited

Altschul, S.F., Madden, T.L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25; p.# 3389-3402.

Amman, R.I., Ludwig, W., and Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cell without cultivation. Microbiol. Rev. 59: p.# 143-169.

Antunes, P., Machado, J., and Peixe, L. 2006. Characterization of antimicrobial resistance and class 1 and 2 integrons in *Salmonella enterica* isolates from different sources in Portugal. J. Antimicrob Chemother. 58; p.# 297-304

Avila P., and de la Cruz, F. 1988. Physical and genetic map of the IncW plasmid R388. Plasmid 20: p.# 155-157.

Baharoglu, Z. Bikard, D., and Mazel, D. 2010. Conjugative DNA Transfer Induces the Bacterial SOS Response and Promotes Antibiotic Resistance Development through Integron Activation. Plos genetic 6; p. # 1-10

Benlloch, S., Acinas, S.G., Antón, J., López-López, S.P., Rodríguez-Valera, L. F. 2001. Archaeal Biodiversity in Crystalliezer Ponds from Solar Salterns: Culture versus PCR. *Microb. Ecol* 41; p. # 12-19.

Cambray, G., Guerot, A., and Mazel, D. 2010. Integrons. Annual Reviews 44:p. 141-166

Chen, C.Y., Wu, K.M., Chang, Y.C., Chang, C.H., Tsai, H.C., Liao, T.L., Liu, Y.M., Chen, H.J., Shen, A.B., and Li, J.C. 2003.Comparative genome analysis of Vibrio vulnificus, a marine pathogen. Genome Res., 13, p. # 2577–2587.

Collis, C. M., Grammaticopoulos, G. , Briton, J., Stokes, H. W and Hall, R. M. 1993. Site-specific insertion of gene cassettes into integrons. Molecular Microbiology 9;p. # 41–52.

Collis, C.M. and Hall, R.M. 1992. Site-specific Deletion and Rearrangement of Integron Insert Genes Catalyzed by the Integron DNA Integrase. J Bacteriol 174; p.# 1574-1585

Díaz-Muñoz, G. Montalvo-Rodríguez, R. (2005). Halophilic Black Yeast *Hortaea werneckii* in the Cabo Rojo Solar Salterns: Its First Record for this Extreme Environment in Puerto Rico. *Caribb. J. Sci* 41, p. # 360-365.

Díaz-Muñoz G. 2006. Fungal Diversity Present at a Hypersaline Environment in Cabo Rojo, Puerto Rico Determined by Characterization of Isolates and Analysis of Environmental rRNA Genes Clones Libraries. M.Sc. Thesis, Department of Biology, University of Puerto Rico, Mayagüez, PR, USA; p .# 140

Dillon, B., L. Thomas, G. Mohmand, A. Zelynski, and J. Iredell. 2005. Multiplex PCR for screening of integrons in bacterial lysates. J. Microbiol. Methods. 62, p.# 221-232.

Elsaied, H., Stokes, H.W., Nakamura, T., Kitamura, K., Fuse, H., and Maruyama, A. 2007. Novel and diverse integron integrase genes and integron-like gene cassettes are prevalent in deep-sea hydrothermal vents. Environ Microbiol, 9; p. # 2298-312

Elsaied, H., Stokes, H.W., Nakamura, T., Kitamura, K., Kurusu, Y.,Kamagata, Y., and Maruyama, A. 2011. Marine integrons containing novel integrase genes attachment sites, *attI*,and associated gene cassettes in polluted sediments from Suez and Tokyo Bays. ISME Journal, 5:p. #1162-1177

Felsenstein, J.1993. PHYLIP (Phylogeny Inference Package), version. 3.6. Seattle: Department of Genetics, University of Washington.

Ferguson,G.P., Totemeyer,S., MacLean,M.J. and Booth,I.R. 1998. Methylglyoxal production in bacteria: suicide or survival? Arch. Microbiol., 170, p. # 209–218.

Fraser,C.M., Gocayne,J.D., White,O., Adams,M.D., Clayton,R.A., Fleischmann,R.D., Bult,C.J., Kerlavage,A.R., Sutton,G. and Kelley,J.M. 1995. The minimal gene complement of Mycoplasma genitalium. Science, 270, p.# 397–403.

Friedrich, M. W. 2002. Phylogenetic analysis reveals multiple lateral transfers of adenosine-5_-phosphosulfate reductase genes among sulfate-reducing microorganisms. J. Bacteriol. 184., p. # 278–289.

Fluit, A.C., and Schmitz, F.J. 1999. Class 1 integrons gene cassette, mobility, and epidemiology. Eur J Clin Microbio Infect Dis. 18; p. # 761-770

Gamieldien, J., A. Ptitsyn, and W. Hide. 2002. Eukaryotic genes in *Mycobacterium tuberculosis* could have a role in pathogenesis and immunomodulation. Trends Genet. 18, p. # 5–8.

Garcia-Quintanilla M, Ramos-Morales F, Casadesus J (2008) Conjugal transfer of the Salmonella enterica virulence plasmid in the mouse intestine. J Bacteriol 190: p. # 1922–1927.

Ghauri, M.A., Khalid, A.M, Grant, S., Grant, W., and Heaphy, S. 2006. High−salt environments and identification of gene cassette asociated open reading frame. Current Microbiology. 52; p. # 487−492

Ghauri, M.A., Khalid, A.M, Grant, S., Heaphy, S. and Grant, W. 2003. Phylogenetic analysis of different isolates of *Sulfobacillus* spp. Isolated from uranium-rich environments and recovery of genes using integron-specific primers. Extremophiles. 7; p. # 341-345

Glover, L. 1971. Geology of the Coamo Area, Puerto Rico and its relation to the volcanic Arc-Trench association. Geological Survey Professional Paper. United States Government Printing Office, Washington.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzelez-Zorn, B., Barbe, J., Marie-Cecile, P., and Mazel, D. **2009.** The Sos Response Controls Integron Recombination. Science 324; p.# 1034

Gopaul, D. N., and G. D. Van Duyne. 1999. Structure and mechanism in site-specific recombination. Curr. Opin. Struct. Biol. 9**:** p. #14–20.

Grainge, I., and M. Jayaram. 1999. The integrase family of recombinase: organization and function of the active site. Mol. Microbiol. 33: p. # 449–456.

Güssow D. and Clackson T. 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Res.*, 17, 4000

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Doson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C. and White, O. 2000. DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. Nature 406, p. # 477- 483.

Hentschel, U., and J. Hacker. 2001. Pathogenicity islands: the tip of the iceberg. Microbes Infect. 3. p**. #** 545–548.

Himmelreich,R., Hilbert,H., Plagens,H., Pirkl,E., Li,B.C. and Herrmann,R. 1996. Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. Nucleic Acids Res., 24,

Igarashi, N., J. Harada, S. Nagashima, K. Matsuura, K. Shimada, and K. V. P. Nagashima. 2001. Horizontal transfer of the photosynthesis gene cluster and operon rearrangement in purple bacteria. J. Mol. Evol. 52, p. # 333–341.

Jiang, S., and Paul, J. H. 1998. Gene transfer by transduction in the Marine Environment. Appl Environ Microbiol. 64; p. # 2780-2787

Koenig, J. E., Boucher, Y., Charlebois, R.L., Nesba, C., zhaxybayeva, O., Bapteste, E., Spencer, M., Joss, M., Stokes, H.W. and Dooittle, W.F. 2008. Integron-associated gene cassettes in Halifax Harbour: assessment of a mobile gene pool in marine sediments. Environ Microbiol, 10; p. # 1462-2920

Konings, W., Sonja-Verena A., Koning, S., and Driessen, A. 2002. The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Annie van Leewenhook 81; p. # 61-72

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., and Woese, C.R. 1996. The ribosomal database project (RDP). Nucleic acids Res 24; p. # 82-85

Marchler-Bauer A., and Bryant, S.H. 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 32; p. # 327-331.

Martinez-Freijo, P. Fluit, A.C., Scmitz, F.J., Grek, JV.S., Verhoef, J., and Jones, M.E. 1998. Class 1 integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J Antimicrob Chemoter 42; p. # 689-696

Mazel, D. 2006. Integrons: agents of bacterial evolution. Nat Rev Microbiol 4; p. # 608-620

Mazel, D. Dychinco,B., Webb, V.A., and Davies, J. 1998. A distinctive class of integron in the *Vibrio cholera* genome. Science 280; p. # 605-608

Mongodin, E.F., Nelson, K.E ., Daugherty, S., DeBoy, R. T. Wister, J., Khouri, H., Weidman, J., Walsh, D.A, Papke, R.T., Sanchez-Perez, G., Sharma, A. K., Nesbo, C. L., Bapteste, E., Doolittle, W. F., Charleboiss, R.L., Legault, B. and Rodriguez-Valera, F. 2005. The genome of *Salinibacter ruber*: Convergence and gene exchange among hyperhalophilic bacteria and archaea. PNAS 102; p. #18147-18152

Montalvo-Rodríguez R., López-Garriga J., Vreeland RH., Oren A., Ventosa A., and Kamekura M. 2000. *Haloterrigena thermotolerans* sp. Nov., a halophilic archaeon from Puerto Rico. Int J Syst Evol Microbiol 50; p. # 1065-1071

Montalvo-Rodríguez, R., Ruiz-Acevedo, A., and López-Garriga, J. 1997. New isolates of extremely halophilic Archaebacteria (Halobacteria) from Puerto Rico and the Caribbean. Caribb. J. Sci. 33; p.98-104

Montalvo-Rodriguez R., Vreeland R., Oren A., Kessel M., Betancourt C., and López-Garriga. 1998. *Halogeometricum borinquense* gen. nov., sp. Nov., a novel halophilic archaeon from Puerto Rico. Int J Syst Bacteriol 48; p. # 1305-1312

Nandi, S. Maurer, J.J. Hofacre, C., and Summers, A.O. 2004. Class 1 antibiotic resistance integrons in poultry litter. PNAS 101; p. # 7118-7122

Nield, B. S., Holmes, A. J., Gillings, M. R., Recchia, G. D., Mabbutt, B. C. Nevalainen, K. M., and Stokes, H. W. 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol. Lett. 195; p.# 59-65

Nemergut, D.R, Martin, A.P. and Schmidt, S.K. 2004. Integron Diversity in Heavy-Metal-Contamined Mine Tailings and Inferences about Integron Evolution. . Appl Environ Microbiol; 70; p. #1160-1168

Nesvera, J. Hochmannova, J., and Patek, M. 1998. An integron of class 1 is present on the plasmid pCG4 from gram-positive bacterium *Corynebacterium glutamicum*. FEMS Micriobiol Lett. 169; p. # 391-395

Nunes-Du¨by, S. E., H. J. Kwon, R. S. Tirumalai, T. Ellenberger, and Landy, A. 1998. Similarities and differences among 105 members of the Int family of site-specific recombinases. Nucleic Acids Res. 26; p. # 391–406.

Ochman, H., Lawrence, J. G., and Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405; p. # 299-304

O'Halloran, F., Lucey, B. Cryan, B. Buckley, T., and Fanning, S. 2004. Molecular characterization of class 1 integrons from Irish thermophilic *Campylobacter* spp. J Antimicrob Chemother 53; p.# 952-957

Oren, A., and Francisco-Rodriguez V. 2001. The contribution of halophilic Bacteria to the red coloration of saltern crystallizer ponds. FEMS Microbiol Ecol 36; p.# 123-130

Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato. 1994. Molecular characterization of an enterobacterial metallo-ß-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. 38; p. # 71-78

Quiñones, F. and Guzmán, S. 1983. Geochemistry of the thermal springs at Coamo, Puerto Rico. Water resources investigations in Puerto Rico and the U.S. Virgin Islands: U.S. Geological Survey. 10, p. #66-72.

Rodríguez-Durán, A. 1998. Nonrandom aggregations and distribution of cave−dwelling bats of Puerto Rico. Journal of Mammalogy 79: p. # 141-146.

Rodríguez-Durán, A. and J. A. Soto-Centeno. 2003. Temperature selection by tropical bats roosting in caves. Journal of Thermal Biology, in press.

Rodríguez-Minguela, C.M., 2005. A Molecular Approach for the Detection and Characterization of Novel Tetracycline Resistance Genes, Integrons, and Integrons-Encoded Antibiotic Resistance Determinants in the Environment. Thesis, Department of Crop and Soil Sciences, Michigan State University, USA.

Rodríguez-Minguela, C.M., J.H.A. Apajalahti, B. Chai, J.R.Cole, and J.M. Tiedje. Integron-encoded integrases are widely distributed across diverse terrestrial and deep sea environment around the globe. (submitted to PNAS).

Rowe-Magnus, D.A., and Mazel, D. 2001. Integrons: natural tools for bacterial genome evolution. Current Opinion in Microbiology; 4 p.# 565-569

Rowe-Magnus, D.A., Guerot, A.M. and Mazel, D. 1999. Super-integrons. Research in Microbiology; 150 p.# 641-651

Rowe-Magnus, Guerout, A.M., Ploncard, P., Dychinco, B., Davies, J., and Mazel, D. 2001. The Evolutionary history of chromosomal super-integrons provides an ancestry for multireistant integrons. PNAS 98; p. # 652-657

Saano, A., Tas, S., Pippola, K., Lidstrom, K., and Van-Elsas, J. 1995. Nucleic acids in the environment: methods and application. Trevors, J.T. and Van Elsas, J. (eds). Berlin: Springer Verlag**.** p.# 49-67

Sabaté, M. and Prats, G. 2002. Estructura y función de los integrones. Enferm Infecc Microbiol Clin, 20; p.# 341-345

Sáenz, Y., Vinué, L., Ruiz, E., Somalo, S., Bezares-Rojo, B., Zarazaga, M., and Torres, C. 2010. Class 1 integrons lacking qacE ¡ and sul1 genes in Escherichia coli isolates of food, animal and humans origen. VetMicrobiol

Satomi M., La Duc M.T. and Venkateswaran K. 2006. *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *International Journal of Systematic and Evolutionary Microbiology*, 56(8): p. #1735-1740.

Sobecky, P.A., and Hazen, TH. 2009. Horizontal gene transfer and mobile genetic elements in marine systems. Methods Mol Biol: 532: p. #435-453

Sobecky, P. A., Mincer, T. J., Chang, A. T., and Helinski, D. R. 1998. Isolation of Broad-Host-Range Replicons from Marine Sediment Bacteria. Appl Environ Microbiol. 64; p. # 2822-2830

Stokes, H. W., and Hall, R.M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. 3; p.# 1669–1683.

Stokes, H.W., Holmes, A.J., Nield, B.S., Holley, M.P. Helena-Nevalainen, K.M., Mabbutt, B.C., and Gillings, M.R. 2001. Gene Cassette PCR: Sequence-Independent Recovery of Entire Genes from Environmental DNA. Appl Environ Microbiol 67; p.# 5240-5246

Stokes, H.W., Nesbo, C. L., Holley, M.,Bahl, M. I., Gillings, M. R. and Boucher, Y. 2006. Class 1 Integrons Potentially Predating the Association with Tn402-Like Transposition Genes Are Present in a Sediment Microbial Community. J Bacteriol. 188; p. # 5722-5730

Sullivan, J. T., S. D. Brown, R. R. Yocum, and C. W. Ronson. 2001. The bio operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. Microbiology 147 :p. # 1315–1322

Sundström L., Rådström, P., Swedberg, G., and Sköld. 1998. Site-specific recombination promotes linkage between trimethoprim-and sulfonamide resistance genes. Sequences characterization of *dhfrV* and *sull* and a recombination active locus of *Tn21*. Mol. Gen. Genet. 213; p. #191-201

Sundström L., and Sköld. 1991. Site-specific insertion of three structural gene cassettes in transposon Tn7. J. Bacteriol. 173; p. # 3025-3028

Sundström L., and Sköld. 1990. The dhfrl trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings. Antimicrob. Agents Chemother. 34; p.# 642-650

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: p.# 1596-1599

Tiedje, J.M. and J.L. Stein. 1999. Microbial Biodiversity: Strategies for its recovery. In A.L. Demain and J. Davies (eds.). Manual of Industrial Microbiology and Biotechnology. Amer. Soc. Microbiol., Washington, D; p. # 682-692

Toussaint, A. and Merlin, C. 2002. Mobile elements as a combination of functional modules. Plasmid, 47; p. # 26-35

Utaker, J. B., K. Andersen, A. Aakra, B. Moen, and I. F. Nes. 2002. Phylogeny and functional expression of ribulose 1,5-bisphosphate carboxylase/oxygenase from the autotrophic ammonia-oxidizing bacterium *Nitrosospira sp*. isolate 40KI. J. Bacteriol. 184; p**. #** 468-478.

van den Burg, B. 2003. Extremophiles as a source for novel enzymes. Curr Opi Microbiol, 3; p. # 213-8

van-Elsas, J. D., and Bailey, M.J. 2002. The Ecology of transfer of mobile genetic elements. FEMS Microbiology Ecology. 42; p. 187-197

Ventosa A., Nieto J., and Oren A. 1998. Biology of Moderately Halophilic Aerobic Bacteria. Microbiol Mol Biol Rev 62; p.# 504-544

Whitman,C.P. 2002. The 4-oxalocrotonate tautomerase family of enzymes: how nature makes new enzymes using a beta-alpha-beta structural motif. Arch. Biochem. Biophys., 402, p. # 1–13.

Xu, H., Davies, J. and Miao, V. 2010. Molecular Characterization of Class 3 Integrons from Delfia spp. J. Bacteriol. 189 (17): p.# 6276-6283.

Young-Kang H., Sook-Jeong, Y., Young-Oh, J., Ho-Tae, S., Hee-Choi, C., Chan-Moon, D., Kil-Lee, W., Chul-Lee, Y., Yong-Seol, S., Taek-Dong, C., and Chul-Lee, J. 2005. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J Antimicrob Chemother 55; p.# 639-644