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CONSTRUCTION OF A CASSETTE FOR CLONING AND ANALYSIS OF REPLICONS

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ABSTRACT: The aim of this work was the construction of a cassette, i.e., a non-replicative molecule formed by linkage of an antibiotic resistance gene and a multiple cloning site. This cassette would allow the cloning and analysis of a wide range of replicons. The *aac* (6')-Ic amikacin gene was isolated and ligated to the multiple cloning site of the pUC18 vector. This construction was *Hind* III digested and cloned in the *Hind* III site of the vector. The resulting pHJ13 clone conferred to the recipient cells the ability to grow in presence of amikacin (cassette marker) and ampicillin (vector gene). By restriction analysis, the cassette orientation was established. Cassette versatility is provided by the presence of the unaltered multiple cloning site segment, and also because it allows sequencing of any replication origin inserted. Cassette functionality was demonstrated by ligation to a replicative region of H plasmid pHH1457. Presence of the *ori* region from pHH1457 and the *aac* (6')-Ic gene was confirmed in *E. coli* transformed clones. The incompatibility properties of the pHH1457 and its capability to replicate in a PolI defective strain were preserved in the pHJII14 construct. Currently, the amikacin cassette is being used in the characterization of H Complex plasmids. **Key Words:** Cassette, H plasmids, replicon, incompatibility.

CONSTRUCCION DE UN CASSETTE PARA EL CLONAMIENTO Y ANALISIS DE REPLICONES

RESUMEN: El objetivo de este trabajo es la construcción de un cassette – molécula no replicativa – formada por un gen de resistencia a un antibiótico y una región de múltiple sitios de clonamiento. Este cassette permitirá el clonamiento y análisis de una amplia variedad de replicones. El gen que determina resistencia a amikacina (*aac* (6')-Ic) fue aislado y ligado a la región de múltiple sitios de clonamiento del vector pUC18. La construcción resultante fue digerida con *Hind* III y clonada en el sitio *Hind* III del vector. El clon pHJ13 resultante confirió a las células receptoras la capacidad de crecer en presencia de amikacina (marcador del cassette) y ampicilina (marcador del vector). Mediante análisis con enzimas de restricción se determinó la orientación del cassette. La versatilidad del cassette se sustenta en la presencia, sin modificaciones, de la región de múltiple sitios de clonamiento, que permitirá obtener la secuencia de nucleótidos de cualquier origen de replicación insertado. La funcionalidad del cassette fue demostrada mediante el ligamiento a una región de replicación del plásmido pHH1457 (Complejo H). La presencia de la región *ori* de pHH1457 y del gen *aac* (6')-Ic fue confirmada en clones de *E. coli*. Las propiedades de incompatibilidad del plásmido H y su capacidad para replicarse en una cepa defectiva en PolI se conservaron en el plásmido pHJII14 construido. El cassette de amikacina está siendo utilizado en la caracterización de plásmidos del Complejo H. **Palabras clave:** Cassette, plásmidos H, replicon, incompatibilidad.

INTRODUCTION

Plasmids are genetic elements of great importance for understanding a variety of genetic phenomena in Procariota. Genetic factors coded in plasmids are dispensable, but they can confer selective advantage for surviving in adverse environments and competing with other microorganisms sharing an ecological niche. Among the genetic factors coded in plasmids are resistance to antimicrobial agents and toxic metals, toxin and bacteriocin production, and ability to degrade unusual compounds. Additionally, plasmid-encoded information can be horizontally transmitted among the population. Consequently, paradigmatic ideas on taxonomy should be modified to explain the presence and activity of the plasmids in bacterial populations.

Replication of plasmids is semiconservative – uni or bidirectional – and independent of the chromosome. The control of replication is carried out by specific negative feedback systems[1,19] depending on the origin of replication of each group of plasmids. Most plasmids contain more than one origin of replication (*ori*) and several regulatory elements located in a DNA fragment no larger than 4 kb. The replicon possesses a gene that codes for a protein or an RNA molecule, which completes the functions of primer in the replication. The Rep protein acts in trans on to specific *ori*, although cis activity has also been reported[1].

The negative control of regulation and random selection of plasmids have a practical consequence: in absence of selective pressure two plasmids with identical replicons can not coexist in the same cell. The phenomenon, known as incompatibility, is caused by random replications of plasmids starting from a common pool, and the hazardous partition during the segregation process. Incompatibility has been used for plasmid classification[10]. Nevertheless, technical and methodological limitations are frequently found, i.e. plasmids under study must encode differential genetic markers so that the development of the offspring may be followed and be transferable by conju-

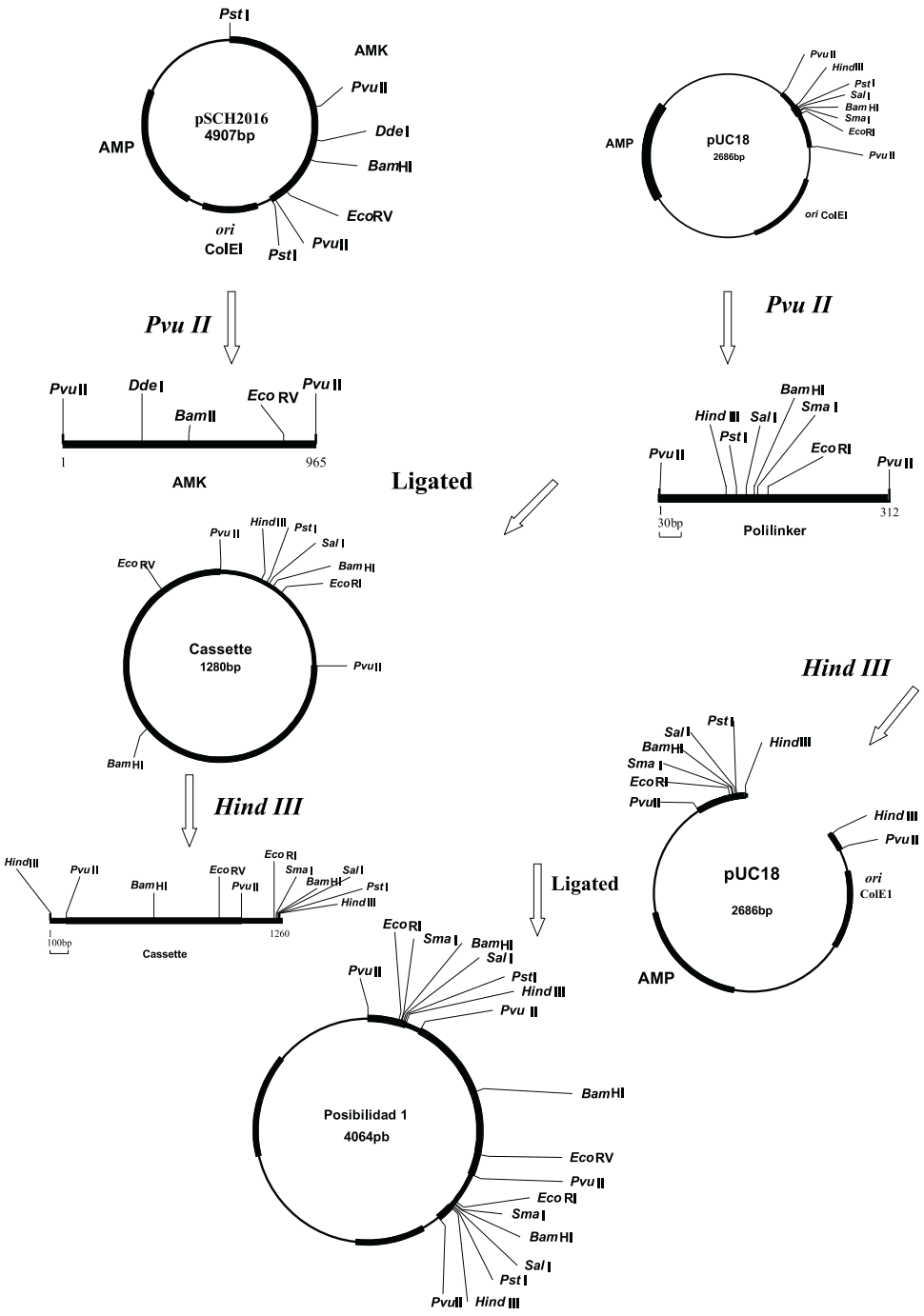

Figure 1. Cassette construction strategy

gation or transformation to a plasmid-free strain. Another strong limiting condition is the presence of more than one replication control system and incompatibility determinants among natural plasmids. So far, 30 incompatibility groups have been established in plasmids transferred to *E. coli* strains.

An alternative and reliable scheme for plasmid classification, technically simpler and less time-consuming, has been developed by Couturier *et al.*[4]. This system, called replicon-typing testing is based on hybridization with specific probes for the incompatibility groups. There are 19 Rep probes available, including those for IncHI1 and IncHI2 subgroups of H plasmids. These Rep probes are of variable size (304 to 2.250 pb), and usually contain not only the genes for the replication control but also other sequences close to the incompatibility determinant.

By classical incompatibility tests, plasmids belonging to the H complex have been classified into two groups: IncHI

Table 1. Plasmids.

Plasmid	Phenotype	Inc.Group	Reference
pHH1457-2	Ap, Cm, Gm, Sm, Km, Tc, Suc, Hg, Te, PacB	HII	15
pMip233	Scr, Te, PacB	HI3	20
pUC18	Ap	ColE1	8
pSBII14	Ap	HII	2
pSCH2016	Ap, Amk	ColE1	18
pHJ13	Amk	Cassette	This work

Antibiotic and metal resistance: Amk, amikacin; Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin; Km, kanamycin; Tc, tetracyclin; Tm, trimethoprim; Scr, sucrose; Hg, mercury; Ars, arsenite; Te, potassium tellurite; PacB, reduction of sensitivity to channel-forming colicins.

and IncHII. DNA-DNA hybridization studies allowed a further separation of IncHI plasmids into subgroups HI1 and HI2. Additionally, plasmid MIP233, exhibiting a strong incompatibility but poor homology with both IncHI1 and IncHI2 subgroups, is included as subgroup HI3. H complex plasmids have been detected in a wide range of bacterial hosts, mostly Enterobacteria[11,15]. IncHII plasmids are compatible with plasmids of the IncHI subgroups, and code for constitutive production of thick flexible pili serologically related to the classical H pili; however, their transfer system is not temperature sensitive[12]. All H plasmids, except for those of the subgroup IncHI1, confer to *E. coli* K12 strains resistance against the lethal action of channel-forming colicins. This property, PacB, appears to be restricted to this group of plasmids[13−17,20]. Plasmids of the H complex are present at a low copy number (1-2 per chromosome). Copy number appears to be controlled by direct repeats in the DNA sequences or iterons located at the origin of replication which, unlike ColE1 plasmid, are independent of DNA polymerase I activity.

Replication of plasmids from the IncHI1 subgroup has been extensively studied and at least three PolI-independent replicons (RepHI1A, RepHI1B and RepHI1A-like) have been identified and cloned[5,6,9]. Meanwhile, nothing is known about the replicative and maintenance determinants of the IncHII group, incompatibility being the only method available for their identification. In order to circumvent the methodological complications resulting from absence of suitable genetic markers, low frequency of transfer, and/or presence of possible multireplicons, studies on the replication region of the H plasmids are in progress in our laboratory[2].

In this work, we report the construction of a cassette useful for cloning and analysis of any replicon origin.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli DH5α was used for plasmid amplification and purification. *E. coli* D110 [F- *thy* M(*rrm*D-*rrn*E) polA λ-] (ATCC N° 35939) was used to select H plasmid autoreplicative regions. To reduce reversions, the polA phenotype was tested before each experiment. Plasmids used in this study are listed in Table 1. All bacterial strains and plasmids are preserved at the Centro Venezolano de Colecciones de Microorganismos (CVCM).

Media and growth conditions

E. coli cells were grown in Luria-Bertani medium. Antimicrobial agents were used at the following concentrations: Ampicillin 120 μgml^{-1}; Amikacin 20 μgml^{-1}; Potassium tellurite 50 μgml^{-1}.

DNA manipulation

Plasmid DNA was isolated by the procedure of Birnboim and Doly[3]. DNA manipulations were done using standard protocols described by Maniatis[8]. Transformation was carried out as described by Hanahan[7]. Restriction endonucleases, T4 DNA ligase, and RNAse were purchased from Promega, and used according to the manufacturer's directions.

When DNA was isolated from single colonies the mini-mini-preps method was used. Shortly, the colonies to be analyzed were picked out with sterile tips, and replicated in LB plus amikacin. The remains of colonies on the sterile tip were placed in a tube containing 10 μl of buffer 1 (Lysozyme 1μg/ml, Tris HCl pH 8.0 50mM and EDTA 50mM). It was incubated by 5 minutes, mixed with 1 ml of phenol pH 5.0, and centrifuged at 12.000xg for 30 sec. The supernatant was electrophoresed on agarose gel.

Cassette construction

The non-replicative fragment of the *aac* (6')-Ic *Serratia marcescens* gene, encoding resistance to amikacin, was isolated from the pSCH2016 plasmid as a *Pvu* II fragment (963 bp)[18]. The multiple cloning site of pUC18 vector was isolated also as a 2t PvuII fragment. Both purified fragments were ligated. The ligation product was *Hind* III digested and cloned in the *Hind* III site of pUC18 vector. The ligation mix was transformed into *E. coli* DH5α and grown in LB supplemented with ampicillin plus amikacin. A recombinant clone pHJ13 (1,300 bp) was obtained and subsequently analyzed.

RESULTS AND DISCUSSION

Construction of the cassette

It is an essential condition for plasmid replication studies, to obtain clones with an unique and active replication origin. The construction and use of cassettes, that is, molecules containing an antibiotic resistance gene and a multiple cloning side, was considered as an adequate

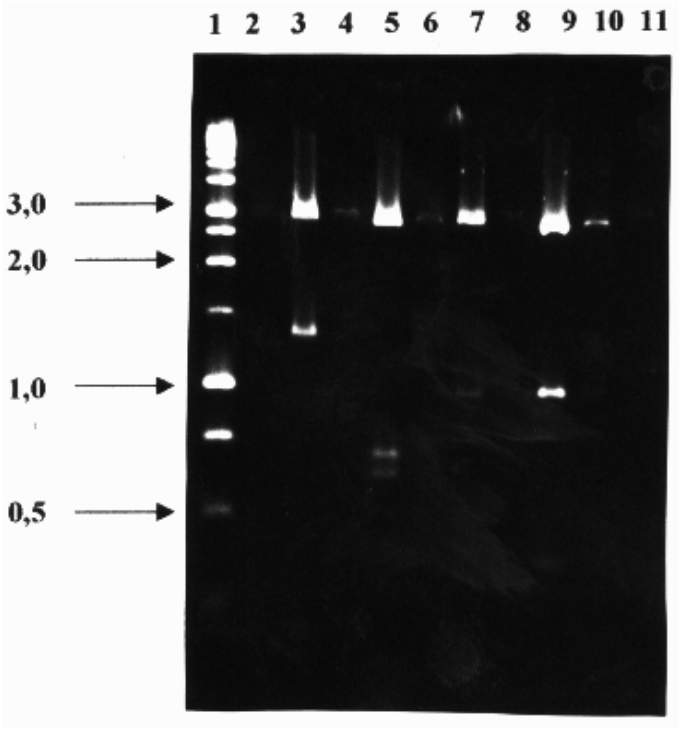

Figure 2. Restriction analysis of random selected clones. The molecular weight standards sizes in kbp are shown on the left side. Lane1: Molecular weight marker. Lane 2: pUC18 × *Hind* III. Lanes 3 and 4: clon × *Hind* III. Lanes 5 and 6: clon × *Bam* HI. Lanes 7 and 8: clon × *Eco* RV × *Hind* III. Lanes 9 and 10: clon × *Eco* RV × *Sal* I. Lane 11: pUC18 × *Hind* III.

strategy. Therefore, to extend our replication studies on plasmids of the H incompatibility group, the construction of a cassette was carried out. In this work, we describe the construction of a cassette by cloning regions from the multiple cloning site and the amikacin *aac* (6')-Ic gene from plasmids pUC18 and pSCH2016. *Pvu* II restriction fragments from both plasmids were purified by electroelution and ligated. Previously, the *aac* (6')-Ic gene was dephosphorylated to increase the binding probability to the multiple cloning site. To maintain the cassette encoding functions, it was cloned in vector PUC18. Enzyme *Hind* III, which cuts in the polylinker region of the cassette, and has no effect on the amikacin gene, was used for cloning. This construct, previously dephosphorylated, was inserted in the *Hind* III site of pUC18 vector. *E. coli* DH5α cells were transformed and grown in medium containing amikacin plus ampicillin. Figure 1 summarizes the cloning strategies.

Analysis of cassette components

E. coli DH5α transformants were selected in amikacin-ampicillin media and analyzed by gel electrophoresis mini-mini-preps. To verify the presence and the orientation of the cassette, random selected clones were restriction analyzed (Figure 2). The enzymes selected were those of the polylinker and the amikacin resistance gene. With *Hind* III (lanes 3 and 4), the presence of two fragments was observed, one of 1.300 bp corresponds to the cassette, and another of 2.670 bp to the linealised pUC18 vector. With *Bam* HI (lanes 5 and 6), three fragments of 508 bp, 527 bp (originated by the *Bam* HI internal site in the amikacin gene) and 2.670 bp (lineal vector) were observed. In double digestions, three fragments were detected. With *Eco* RV and *Hind* III (lanes 7 and 8) 252 bp, 823 bp (originated by the *Eco* RV site in the amikacin gene) and 2.670 bp (lineal vector) bands were observed. To confirm the cassette orientation, double restrictions were carried out with *Eco* RV and *Sal* I (lanes 9 and 10), and bands of 222 bp, 843 bp and 2.670 were observed. With these results we constructed the restriction map shown in Figure 3, where the orientation of the cassette is indicated. A recombinant clone, pHJ13, was selected for further studies. It contains an insert cassette-type that includes the amikacin resistance gene and the multiple cloning site. These analyses allow us to conclude that the orientation of the primers annealing sites would allow the subsequent sequencing of any origin of replication. The amikacin resistance gene is flanked by the polylinker at both ends allowing scission of the cassette by any one of the restriction sites at the polylinker, and conserving the primer annealing sites.

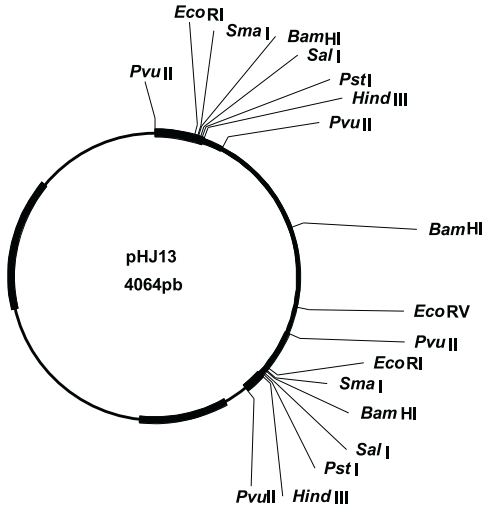

Figure 3. Restriction map of pHJ13.

Testing the cassette functionality

To test the cassette functionality it was used to clone the origin of replication from plasmid pHH1457. This plasmid was selected because it has been extensively studied and its origin of replication has been previously cloned in our laboratory[2]. The cassette was used for cloning a DNA fragment (1,400 bp) containing the *ori* region from pHH1457 (IncHII). *E. coli* DH5α transformants were selected in

medium plus amikacin. Amikacin resistant colonies were randomly selected and analyzed by mini-mini-preps. A transformant, pHJII14, was selected and further studied[2]. The presence of the parental pHH1457 origin ligated to the cassette was confirmed by restriction analysis. Two fragments corresponding to the antibiotic resistance gene, without replicative properties, and to the region of the pHH1457 origin were identified. H Complex plasmids are regulated by iterons and do not require DNA Polymerase I for replication. To verify that the cloned *ori* of pHH1457 does not require PolI for replication, the construct was used to transform *E. coli pol*A cells. Analysis of amikacin resistant colonies showed presence of the construct. These results were confirmed by restriction analysis. It was also demonstrated that the construction maintains the incompatibility properties of pHH1457[2].

The above results demonstrated that the *aac* (6')-Ic cassette is an adequate vehicle for analysis of replicons. That is, it ensures that plasmids constructed are able to replicate by the cloned region. Furthermore, the presence of multiple cloning sites introduces a great versatility because of the wide range of restriction enzymes that can be used for cloning. The presence of sequencing primers annealing sites allows sequencing of any cloned region.

Since classical incompatibility tests used in plasmid classification are laborious and the results are not always reliable, another methodological approach has been developed, i.e. DNA probes containing genes for replication. So far, about 20 rep probes have been constructed and successfully used for plasmid identification. Nonetheless, Rep probes for the remaining groups are not available. Therefore, it is thought of interest to use the cassette for cloning and characterization of the remaining incompatibility groups, including those of the H complex. The knowledge of the molecular basis of incompatibility, stability and replication of the H plasmids may help to understand factors involved in prevalence and dissemination of these plasmids among different genera of bacteria.

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