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Option Biotechnology

Diploma 2007

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Identification of the genetic elements involved in pediocin PD-1synthesis

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Titre / *Titel*:

Identifizierung der für die Pediocin PD-1 Synthese notwendigen Gene

Description / *Beschreibung*:

Pediococcus damnosus DSM20331 produziert das antimikrobielle Peptid Pediocin PD-1, das gegen eine Vielzahl gram-positiver Bakterien aktiv ist. Im Rahmen eines Forschungsprojektes konnten wir die Sequenz des Gens *pedA*, das für Pediocin PD-1 codiert, bestimmen. Diese erlaubte uns das Peptid der Gruppe der Lantibiotika zuzuordnen.

Im Rahmen dieser Diplomarbeit sollen nun die restlichen Gene identifiziert werden, die für die Synthese dieses antimikrobiellen Peptids notwendig sind. Aufgrund der bereits in der Literatur beschriebenen Lantibiotika-Synthesewegen kann man davon ausgehen, dass auch die Gene für die Synthese von Pediocin PD-1 vermutlich als Gencluster organisiert sind und sich unmittelbar neben dem *pedA*-Gen befinden. In der Semesterarbeit von Mirko Ledda (2007) konnte gezeigt werden, dass sich das *pedA* Gen auf einem grossen Plasmid (50 – 100 kb) befindet.

Folgendes Vorgehen wird vorgeschlagen:

- Auftrennen der Plasmide mittels PFGE und anschliessender Extraktion aus dem Gel
- Erstellen einer Plasmid-DNA-Genbank: Restriktionen mit verschiedenen Endonukleasen und Subklonierung der Fragmente in den Vektor pUC18
- Identifizieren des *pedA* enthaltenden rekombinanten Plasmids: Screening mittels PCR und/oder Hybridisierung mit einer *pedA* Sonde; Lokalisierung der *pedA* Region auf dem Insert
- Sequenzieren der *pedA* flankierenden Regionen und identifizieren der ORFs

Objectifs / *Ziele:*

- Bestimmung der Sequenzen der Gene aus *Pediococcus damnosus*, die für die Synthese von Pediocin PD-1 notwendig sind.

Signature ou visa / *Unterschrift oder Visum*

Leiter Vertiefungsrichtung Biotechnologie .....

Professeur/*Dozent*: Sergio Schmid .....

Etudiant/*Student*: .....

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Identification of the genetic elements involved in pediocin PD-1 synthesis

Identification des éléments génétiques impliqués dans la synthèse de pédiocine PD-1

Aim: Confirm the presence of *pedA* gene, known part of pediocin PD-1 operon, in one of *Pediococcus damnosus* plasmids. Construct a plasmid library using this isolated plasmid, digested with endonucleases and subcloned in pZErO-2 vector. Identify recombinant plasmids containing *pedA* gene by PCR screening or/and by hybridization with *pedA* probe. Sequence the insert and localize *pedA* region in order to identify the remaining part of the insert, used to expand pediocin PD-1 operon known sequence. Identify ORF on this sequence.

Results: *pedA* gene has been confirmed in one of *P.damnosus* plasmid but its purification gave weak DNA concentration. A plasmid library composed of 160 clones has been created using this purified plasmid, digested with *Eco*RI and *Hind*III; no *pedA* positive recombinant plasmids have been found.
Another plasmid library composed of 82 clones has been built and stored using total *P.damnosus* plasmid DNA, digested with *Eco*RI; three *pedA* positive recombinant plasmids have been found (p60, p70 and p76). Genetic maps of these plasmids show they possess the same insert with an estimated size of 3300 bp. p60 DNA sequencing shows no *pedA* region but could be one of *P.damnosus* plasmids partially homologous to pF8801. A 3rd plasmid library, composed of 63 clones, has been built and stored using total *P.damnosus* plasmid DNA, digested with *Hind*III, not yet analyzed.

Key words: Pediocin PD-1*, Pediococcus damnosus*, Bacteriocins.

Objectif: Confirmer la présence du gène *pedA*, partie de l'opéron de pédiocine PD-1 connue, dans un des plasmides de *Pediococcus damnosus*. Construire une banque plasmidique en utilisant ce plasmide isolé, digéré avec des endonucléases avant d'être cloné dans le vecteur pZErO-2. Identifier les plasmides recombinants contenant le gène *pedA* par criblage PCR ou/et par hybridation avec une sonde *pedA*. Séquencer l'insert et localiser la région *pedA* afin d'identifier la partie de l'insert restant qui permet d'étendre la partie connue de l'opéron PD-1. Trouver l'ORF de cette séquence.

Résultats: Le gène *pedA* a été confirmé dans un plasmide de *P.damnosus* mais sa purification a donné une très basse concentration d'ADN. Une banque plasmidique, composée de 160 clones a été construite à partir de ce plasmide digéré avec *Eco*RI et *Hind*III ; aucun plasmide recombinant positif n'a été trouvé.
Une autre banque plasmidique, composée de 82 clones, a donc été construite, et stockée, avec l'ADN plasmidique de *P.damnosus* digéré avec *Eco*RI; 3 plasmides recombinants positifs ont été trouvés (p60, p70 et p76). La carte génétique de ces trois plasmides indique le même insert d'environ 3300 bp. L'insert de p60 a été séquencé et ne présente aucune région *pedA* mais pourrait être un plasmide de *P.damnosus* similaire à pF8801. Une 3ème banque plasmidique a été créée, et stockée, avec l'ADN plasmidique de *P.damnosus* digéré avec *Hind*III, pas encore analysée.

Mots-clés: Pédiocine PD-1*, Pediococcus damnosus*, Bactériocines.

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1 Introduction

1.1 Aims

The goal of this diploma work was to identify the sequence of the operon from *Pediococcus damnosus* coding for pediocin PD-1. To do so, different molecular biology techniques had to be used in order to achieve the isolation of this gene and its sequencing.
A part of this operon, the *pedA* gene, has already been sequenced in a previous project (Bonvin, 2006) and its location was detected in one of *P. damnosus* plasmids (Ledda, 2007). The location of *pedA* gene in the genome had to be confirmed before going any further with this diploma work. Once this assignment realized, the plasmid containing *pedA* had to be isolated, digested and inserted into a bacterial vector. These recombinant plasmids have then to be inserted into *E.coli* cells to create a plasmid library. An identification of the recombinant plasmid had to be achieved by PCR screening or by hybridization with a *pedA* probe to find *pedA* positive clones. Finally, an extraction of the *pedA* positive recombinant plasmids and a specific digestion had to be carried through in order to create their genetic map. An extern service is then used to sequence part of PD-1 operon isolated. In addition, the Open Reading Frame (ORF) had to be identified with bioinformatics tools.

1.2 Theoretical introduction

Nowadays, consumers are consistently concerned about possible adverse health effects that may be caused by the presence of chemical additives in food. This aspect coupled with the increasing demand for minimally processed long shelf life foods has stimulated research interest in finding natural efficiencies preservatives. Bacteriocins, produced by lactic acid bacteria (LAB), may be considered as biopreservatives that fulfill these requirements (Chen *et al.*, 2003). The term biopreservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life (Schillinger *et al.*, 1996). These bacteriocins can be exploited in foods to replace chemical preservatives. However, in order to fully realize their potential, it is necessary to understand the biology of bacteriocins: their production, immunity and mode of action have to be first elucidated.

This is the reason why a diploma work has been achieved on the isolation of the geneti elements involved in pediocin PD-1 synthesis. PD-1 being a bacteriocin produced by lactic acid bacteria *Pediococcus damnosus*. Once this operon sequenced, it will be possible to transfer it into a strain of *Escherichia coli* to allow a simple manipulation of the gene in preparation for its production by *Pichia pastoris* and its practical manipulation. Pediocin production can be then used in wine, beer and other fermented food and beverage products to prevent the growth of unwanted microbes. It could replace the excessive use of sulphur dioxide, the only authorized agent for microbial stabilisation and control in must and wine. This chemical compound added in these beverages holds various disadvantages for the quality of the end-products and is confronted by mounting consumer resistance (Schoemann, 1999). Synthesis of PD-1 could then be a method to control malolactic bacteria in wine and provide an alternative to chemical preservatives (Bauer *et al.*, 2003). In a purified form bacteriocins are colourless, tasteless and odourless. Furthermore, they are non-toxic and safe for human consumption. This is why it would be the perfect answer in the future for a natural solution to sulphur dioxide in wine.

1.2.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are gram-positive usually non-motile, non-spore-forming rods and cocci that are associated by their common metabolic and physiological characteristics. Lactics are classified by the fermentation pathway used to ferment glucose and by their specific cell morphology. The genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* are important members of this group. Their metabolism is fermentative; some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. The lactic acid bacteria belong to two main groups: the homofermenters and the heterofermenters that differ for their lactic acid production.

- Homofermenters produce lactic acid via the glycolytic pathway.
- Heterofermenters produce lactic acid plus ethanol, acetate and carbon dioxide via the pentose/phosphate pathway.

Table 1: Major lactic acid bacteria in fermented plant products (Beuchat, 1995)

Homofermenter	Facultative Homofermenter	Obligate Heterofermenter
Enterococcus faecium *Lactobacillus lactis* *Streptococcus bovis* ***Pedicoccus damnosus*** *Pediococcus pentocacus*	*Lactobacillus bavaricus* *Lactobacillus casei* *Lactobacillus curvatus* *Lactobacillus plantarum* *Lactobacillus sake*	*Lactobacillus brevis* *Lactobacillus confusus* *Lactobacillus fermentatum* *Lactobacillus buchneri* *Leuconostoc dextranicum*

These bacteria are broadly used in the production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.) and sausage. The industrial importance of the LAB is further evidenced by their generally regarded as safe (GRAS) status by the World Health Organisation (WHO). In addition to their ability to produce desirable fermented food, several strains produce proteinaceous bacteriocins as *Pediococcus damnosus*.

1.2.2 Bacteriocins

Bacteriocins are antimicrobial proteins that are produced by lactic acid bacteria, which kill or inhibit the growth of other bacteria. These structurally, functionally, and ecologically diverse peptides are ribosomally synthesized (Klaenhammer, 1993). They have potential practical applications and a great number of diverse bacteriocins have been identified and characterised in recent years. Bacteriocins are encoded by genes usually organized in operon cluster (Nes *et al.,*1996). These bacteriocin gene clusters can be located on the chromosome (Altena and al,. 2000), in a plasmid (Engelke *et al.*, 1992) or in transposons.

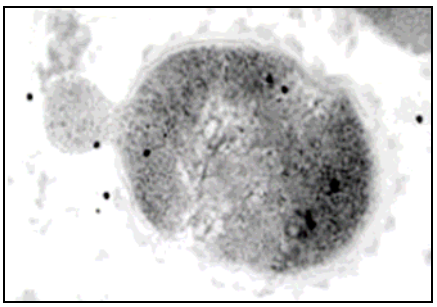

Figure 1: *Cellular lysis caused by the action of a bacteriocin.*

Bacteriocins are often confused in the literature with antibiotics (Hansen 1993), but are clearly distinguishable from clinical antibiotics, as shown in Table 2.

Table 2: Differences between bacteriocins and antibiotics (Klaenhammer, 1993)

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Second metabolite
Activity	Narrow spectrum	Varying spectrum
Resistance or tolerance	Membrane composition	Depending on the mode of action
Host cell immunity	Yes	No
Mode of action	Mostly pore formation, but in a few cases cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity	None known	Yes

The classification of bacteriocins is commonly divided into three groups, as summarized in Table 3. The first class is the lantibiotics which are small peptides containing the amino acids lanthionine (Lan), β-methyllanthionine (MeLan) and a number of dehydrated amino acids. Class I is further subdivided into type Ia and type IIb according to their chemical structures and antimicrobial activities. Type Ia consists of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid class Ib. This type Ib regroups globular bacteriocins with net negative charge (Altena *et al.,* 2000). About class II, the largest group of bacteriocins in the classification system, they are small heat-stable, non-Lan-containing and membrane active peptides. Type IIa includes pediocin-like peptides and has attracted much attention due to their activity against *Listeria*. Concerning type IIb, it contains bacteriocins requiring two different peptides for their activity. The members of class III are larger (>30 kDa), heatlabile proteins but are not very well characterized because they are of lesser interest for food scientifics. A class IV, which would consist of bacteriocins that form large complexes with other macromolecules, have been proposed (Klaenhammer, 1993) but no such bacteriocins have yet been characterized adequately at the biochemical level.

Table 3: Summary of the classification of bacteriocins (Klaenhammer, 1993).

Group	Class	Features	Examples
I	Ia	Lantibiotics, small (< 5 kDa) peptides	Nisin
	Ib	Lantibiotics, globular peptides with no net charge or net negative charge	Mersacidin
II	IIa	Small heat-stable peptides	Pediocin PA-1
	IIb	Two components required for activity	Lactococcins G
III		Large molecules	Helveticincs J

1.2.3 Lantibiotics

Pediocin PD-1 is not yet fully characterized but it seems to belong to lantibiotics. This bacteriocin group, produced by Gram-positive bacteria, is unique in that they are produced on the ribosome as a prepeptide that undergoes an extensive post-translational modification to form the biologically active peptide. Approximately 50 lantibiotics are known (Patton, 2005) and have various size and structures that create different antibiotics activities.

Concerning lantibiotic biosynthetic gene, the generic locus symbol created is *Lan*, with a more specific name for each member of this family (e.g. *ped* for pediocin). For genetic regulation of lantibiotics, the operon contains generally genes coding for the prepeptide (*LanA*, *pedA* for PD-1), enzymes responsible for modification reactions (*LanB,C* or *LanM*), processing proteases responsible for removing the leader peptide (*LanP*), regulations proteins (*LanR,K*) and proteins involved in producing immunity (*LanI*) (Schnell *et al.*, 1992).

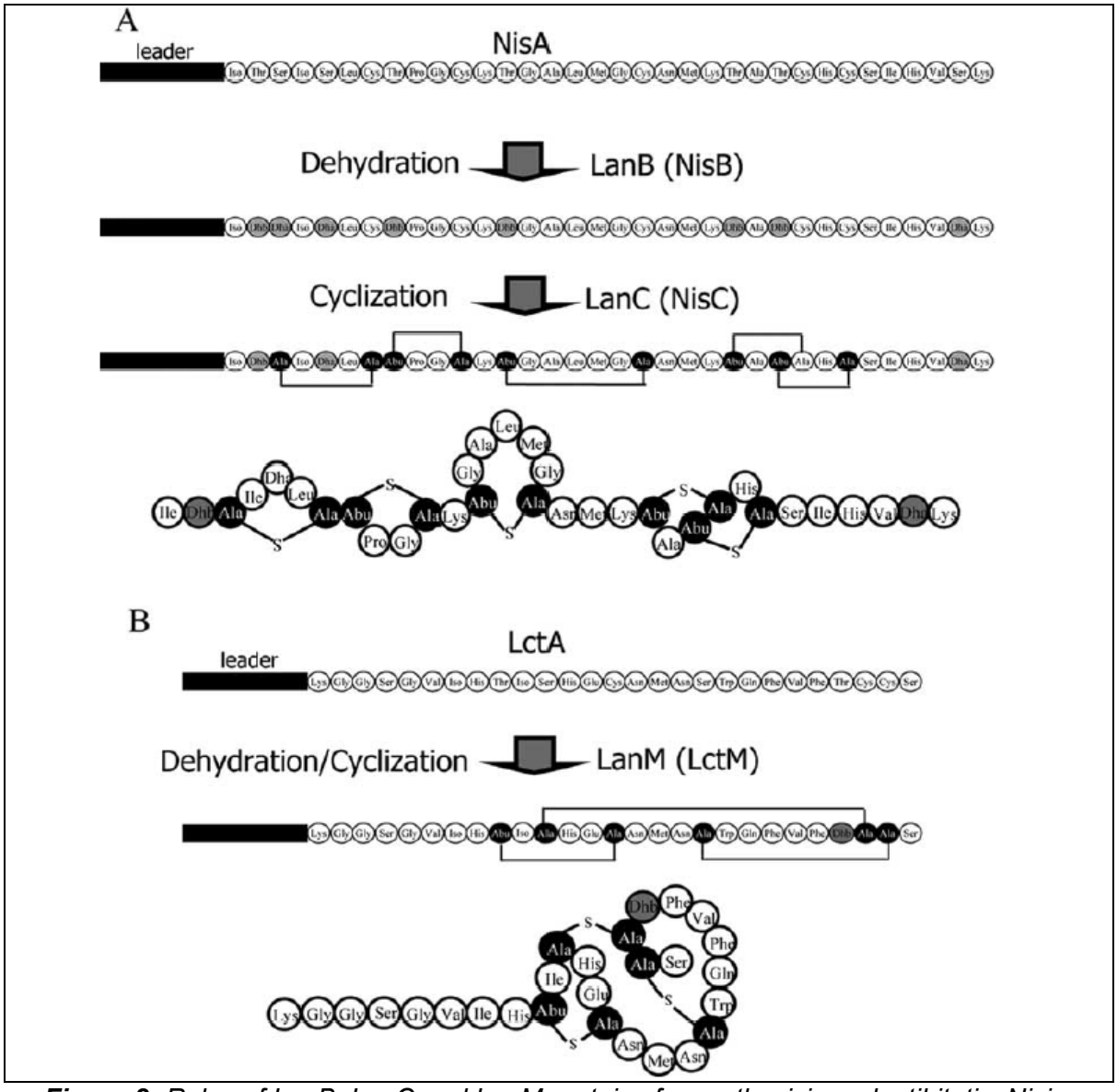

Figure 2: *Roles of LanB, LanC and LanM proteins for synthesising a lantibitotic, Nisin.*

Both types Ia and Ib lantibiotics kill target cells by interrupting cell wall synthesis through high-affinity binding to the lipid II molecule, a precursor molecule that plays an essential role in the synthesis of the peptidoglycan layer. Type Ia lantibiotics are also able to kill bacteria by an additional mechanism: binding to the lipid II molecule and thereby forming pores in the cytoplasmic membrane of the target.

1.2.4 Pedioccocus damnosus

Pediococcus is a genus of Gram-positive lactic acid bacteria, placed within the family of Lactobacillaceae that are purely homofermentative. They are commonly found in fermented vegetables, in dairy products and in meat. Although eight species of *Pediococcus* were listed, more recent information indicates that only five species belong to the genus: *P.acidilactici, P.dextrinicus, P.parvulus, P. pentosaeus* and of course, *P.damnosus*. Little research has been done on these bacteriocins despite they cause spoilage in beer, wine and other high-pH food products. However, several bacteriocins have already been described, as pediocin A and St18 from *P.pentosaceus*, pediocin PA-1/AcH from *P.acidilactici* and of course, pediocin PD-1 from *P.damnosus*.

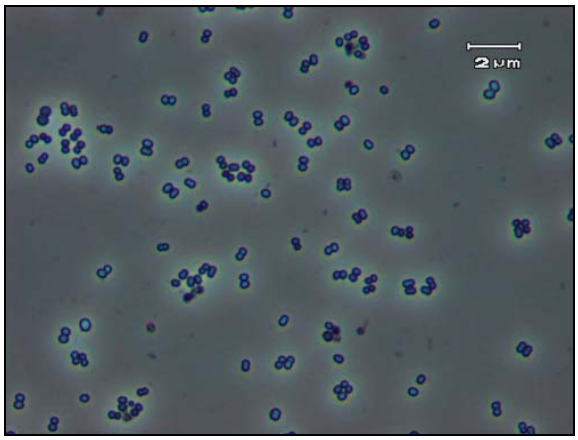

Figure 3 : *Pediococcus 1000x*

1.2.5 Pediocin PD-1

Pediocin PD-1, produced by the lactic acid bacteria *Pedioccocus damnsosus*, is active against a range of Gram-positive bacteria, including members of the genera *Clostridium, Bacillus, Staphylococcus, Leuconostoc* and the species *Oenococcus oeni*. This antimicrobial peptide PD-1 differs from other pediocin-like bacteriocins in that it is not active against pediococci and has a unique sensitivity pattern to proteolityc enzymes. This hydrophobic peptide is 2866.87 ± 0.4 Da in size, resistant to heat (10 min at 121°C) and remains active after 30 min of incubation between pH 2.0 and 10.0 (Green *et al.,* 1997). It has an isoelectric point of ca. 9.0. It has been shown that the pediocin PD-1 sequence is substantially homologous, up to 93 %, to plantaricin C, a lantibiotic produced by *Lactobacillus plantarum* 441 (Bauer *et al.,* 2005).

The primary mode of action of pediocin PD-1 is most probably due to pore formation. When used againt *O. oeni*, it is suggested that PD-1 acts on the cytoplasmic membrane of these bacteria. However, PD-1 may also inhibit cell wall biosynthesis which leads to cell lysis. It has been shown PD-1 is more effective in removal of *O. oeni* from stainless steel surfaces in Chardonnay must than nisin and plantaricin 423 (Bauer et *al*., 2004). Pediocin PD-1 production can be stimulated by growth factors such as peptone, $MnSO_4$ and Tween 80 (Nel et *al*., 2001).

1.3 Practical introduction

To achieve this diploma work, several molecular biology techniques had to be used in order to characterize the genetic elements needed for the synthesis of pediocin PD-1. However, part of pediocin PD-1 operon has already been sequenced in in a previous work (Bonvin, 2006): the *pedA* gene, encoding the amino acids precursor of the peptide pediocin PD-1. Its sequence is shown in Figure 4.
It seems that the operon of PD-1 is organized in gene cluster, as in lantibiotics. Therefore, the rest of the operon would be near the *pedA* gene; it is then possible to use this knowledge to expand the known sequence until the entire operon encoding for pediocin PD-1 is found.

```
  1 ATAGCCATAT TTCATCTTTT TATCTCCTTA CTTATGTCAT TAAGTAATGA CACGGTTCTA
 61  ACCCTTTAAT TATACAGTAT CAAAAAAGAG GCCACAACTG TTAAGATGTG ACACAATCAT
121 TAAAATTTGA TTTTTATTAA GAAATAGTAT ACGAAACATT GAAGGCTCAA AAATTTTCTG
181 GAAATTCCCC TCAATTTAAT CCACTTTTAC CATATAACTT GTTTACTTCA CTTGAAAGGG
241 AATAATTTTG GAACTAAAAT GAAATTGTTC ACAAATATGC AGATAGGAGG ATTATTTATA
301 GTTTGAAGCA GCCCTAATCA CAAAGTGTAC CTCATTTACA ATCTGAAAAG GAGACGTGAG
361 TTGTTATGAA GAAAAATTTA ATGAAGTCCG CAGAAGAATC ATCAGGTAAT GTTTTGGAAG
421 AATTAAATAA TGCACAGCTT GGTATGATTA GTGGTGGTAA GAAGATCAAG AAGAGTTCTA
481 GTGGTGACAT TTGTACCTTA ACAAGTGAAT GTGATCATTT GGCAACATGG GTATGTTGCT
541 AAAAATCATT TCATGACTAT TTTTTGCTAT TAATAATGAG TGATTAGGGT TGCTTCAAGC
601 TATAAATTCT AAATTGATCA CATGGTAAAA AAAACAGATT CCGTGCAGGG CCAAATTGAA
661 AAAGCAATAG GGACAGATGT ATTACAGCTG GCTATGGATA ATTTAAGTAA GTTC
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Figure 4: *Known DNA sequence of pediocin PD-1 (714 bp)*
Potential –35/-10 site, Potential RBS and Potential start codons

On this *pedA* gene, it is assumed that a ribosome binding site (RBS) should be located on position 346-354. It is followed by three potential start codons; it has been deduced that the real start codon for the prepeptide is one of the first two, on position 366-368 for the first and 381-383 for the second, since the third start codon, on position 444-447, would furnish a too little prepeptide. Another RBS could be located on position 368-376, but this would exclude the first start codon.
The location of this *pedA* gene has been detected in one of *Pediococcus damnosus* plasmids (Ledda, 2007). This affirmation had first to be confirmed before going any further within this project. To do so, different molecular techniques are used to identify whether *pedA* is part of the chromosome or of one of the plasmids from the bacteria. The very-high-molecular-weight DNA molecules from *P.damnosus* bacteria have first to be separated by Pulsed-Field Gel Electrophoresis (PFGE). These DNA fragments are then transferred on a Hybond-N+_nylon membrane by Southern blotting. Hybridization with *pedA* probe is then realized in order to detect which DNA fragments contains the *pedA* gene.
Once the location of *pedA* found and its presence in a plasmid confirmed, it is possible to extract the desired plasmid from the Pulsed-Field gel. This plasmid is then digested and ligated into a bacterial vector, pZErO-2, containing a resistance to kanamycin antibiotic. The recombinant plasmids are then transformed by electroporation into *E.coli* TOP10 cells and a screening of these transformed cells on LB-kanamycin plates is realized. It allows to isolate every cell that possesses kanamycin resistance gene and to form a plasmid bank. DNA is then extracted from these transformed cells and a PCR screening is performed to detect which cell contains the recombinant plasmid with the desired *pedA* gene. This plasmid is then digested and its insert is sequenced to expand pediocin PD-1 known sequence.

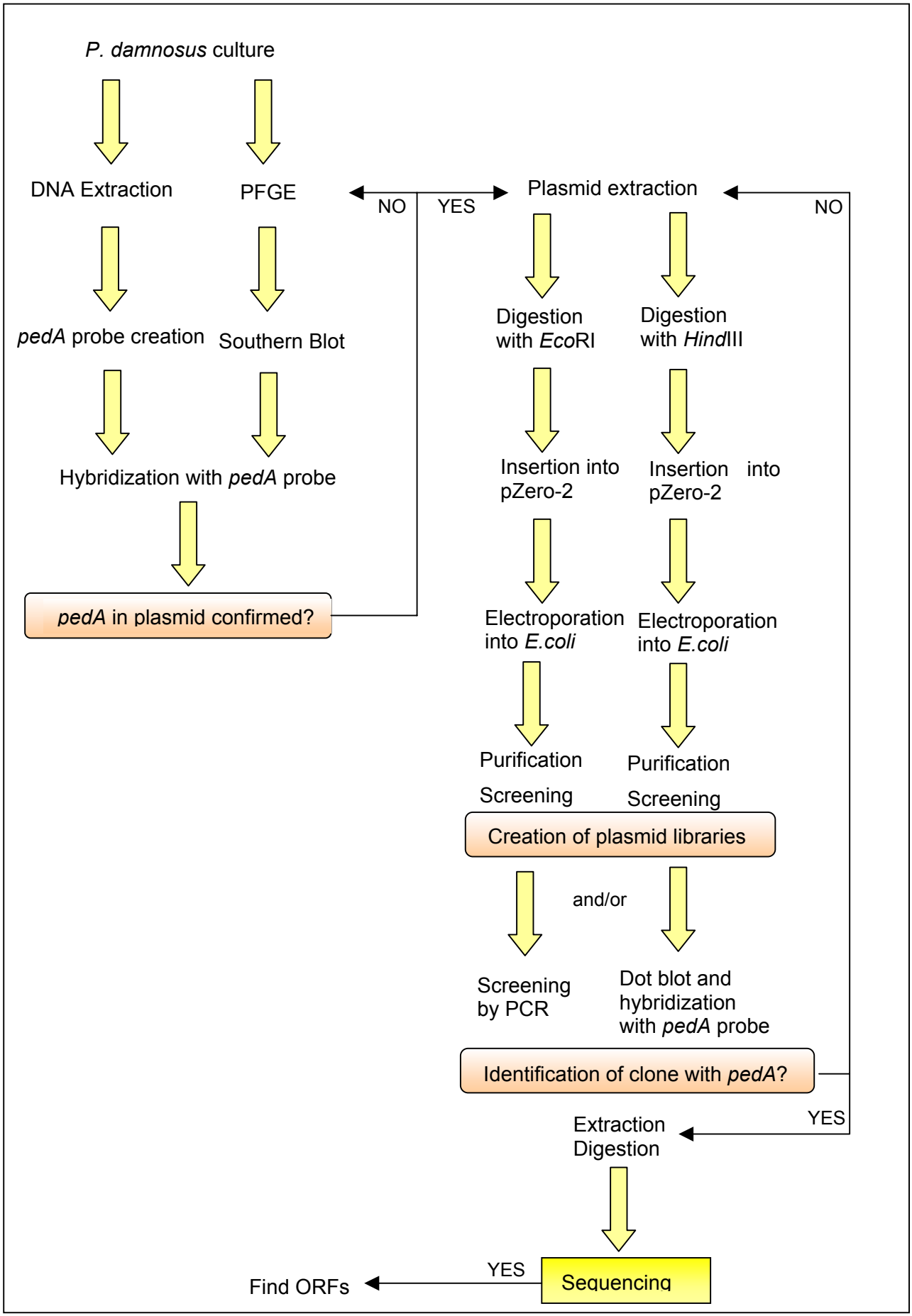


Figure 5: *Summary of the steps needed for the characterization of the PD-1 operon*

1.3.1 Pulsed-Field Gel Electrophoresis

To confirm the location of the *pedA* gene in one of *P.damnosus* plasmids (Ledda, 2007), the Pulsed-Field Gel Electrophoresis (PFGE) system is used; it has the advantage to work directly with very-high-molecular-weight DNA molecules. A separation of the different DNA molecules is then possible by the use of an alternating electrical field as shown on Figure 6. By changing the direction of the electric field frequently, much greater size resolution can be obtained. Indeed with continuous field electrophoresis, DNA molecules above 25 kb migrate with the same mobility regardless of size. PFGE is thus a solution to separate DNA molecules several million bases in length since with each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than larger DNA molecules (Ausubel, 2000). However, very-high-molecular-weight molecules are extremely fragile and have to be prepared and manipulated while embedded in agarose blocks in order to avoid shearing forces.

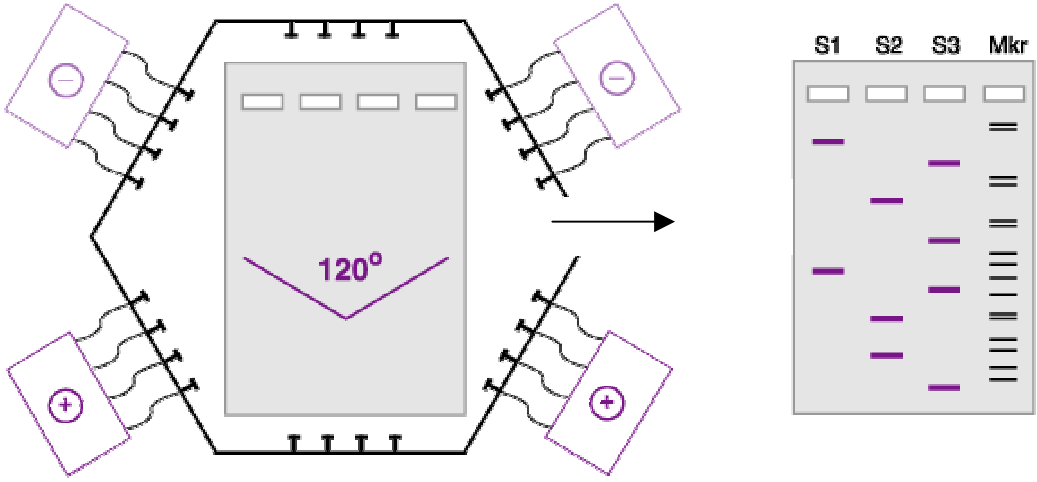


Figure 6: *Pulsed-Field Electrophoresis Gel illustration*

On Figure 6, the schematic explanation of the PFGE is shown for a contour-clamped homogeneous electric field system (CHEF-DR® III) used for this diploma work. This technique changes the direction of the electric field to reorient the DNA by changing the polarity of an electrode array. DNA separation with better resolution, speed and accuracy is obtained with CHEF-DR® III than with initial pulsed field techniques (BioRad, 1992).

1.3.2 Southern Blot

Southern blotting, created by Edward M. Southern in 1975, is designed to transfer DNA fragments from an electrophoresis gel to a membrane support via upward capillary. A high-salt transfer buffer is used to promote binding of DNA to the membrane. After the final immobilization by UV irradiation or baking, the DNA can be subjected to hybridization analysis in order to locate a particular sequence of DNA within a complex mixture (Ausubel, 1999). For this diploma work, southern blotting is used to transfer the size-separated DNA from the Pulsed-Field Electrophoresis gel to a membrane support; DNA fragments retain the same pattern of separation they had on the gel. The membrane chosen for this Southern Blot is a nylon membrane since it is relatively robust and can be reprobed several times. With a high-salt buffer, DNA is not permanently immobilized and UV irradiation is needed if a nylon membrane is used.

1.3.3 Hybridization with a pedA probe

Once the Southern Blot realized, hybridization with a *pedA* probe can be performed to localize the *pedA* gene on the membrane. The hybridization probe is in fact a fragment of DNA of variable length (usually 100-1000 bases long) used to detect, in target DNA samples, the presence of nucleotide sequences that are complementary to the sequence in the probe. An enzyme is added to the probe so that, when adding the correct substrate, a chemiluminescent label is obtained as illustrated on Figure 7.

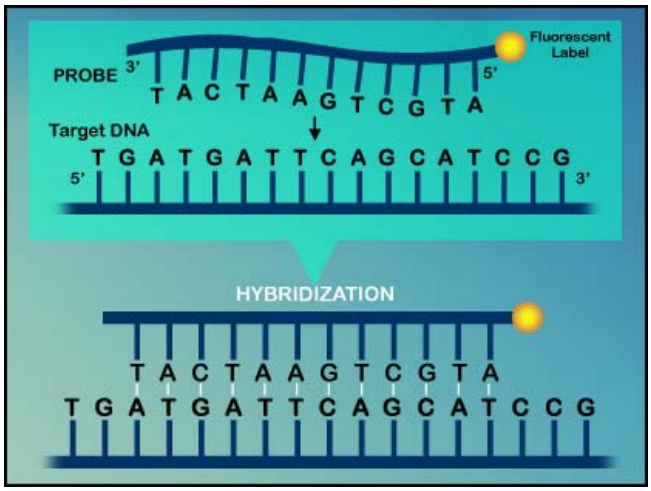


Figure 7: *Example of a hybridization*

For this diploma work, the probe is created from the known *pedA* sequence with PCR-amplification using primers pedR and pedF. Localization of these primers on the *pedA* sequence and the PCR product are illustrated on appendices *8.3* and *8.4*. Alkaline phosphatase is added to this probe to produce light when the correct substrate is added. This step makes possible the localization of DNA samples containing *pedA* gene and therefore, the pediocin PD-1 operon. The substrate used with alkaline phosphatase is CDP-Star®. It is a chemiluminescent substrate, its scientific name is Disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.13,7]decan}-4-yl)-1-phenyl phosphate. It has a molecular weight of 496 Da. CDP-Star reagent produces a light signal when it is activated by alkaline phosphatase, accumulates in its dephosphorylated form and decomposes at a constant rate for up to several days. Light production resulting from chemical decomposition exhibits an initial delay of 1-2 hours followed by a persistent glow that lasts as long as there is free substrate available. The glow signal may endure for hours or even days if signal intensity is low (Applied Biosystems, 2001).

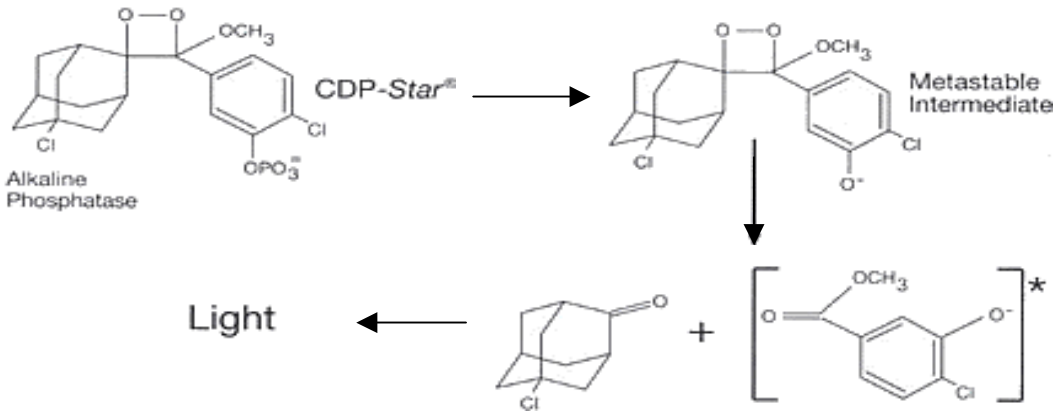
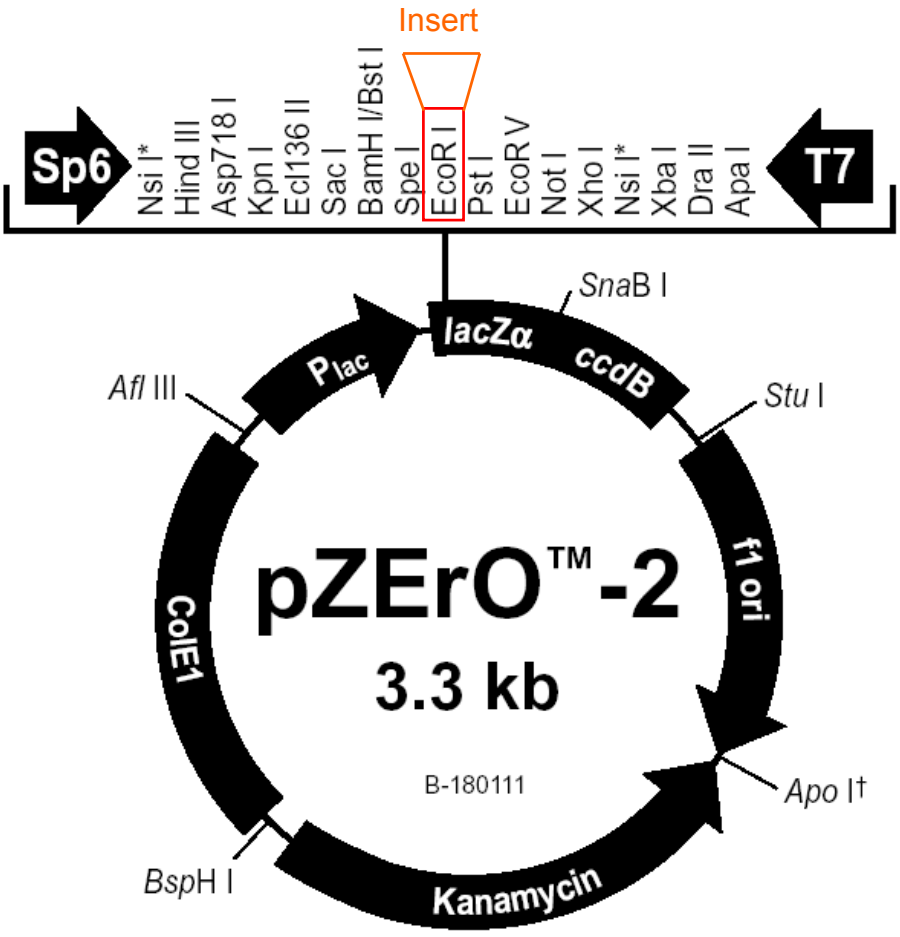


Figure 8: *Light emission mechanism of CDP-Star® substrate with alkaline phosphatase.*

1.3.4 Cloning into pZErO™-2 vector

P.damnosus plasmid, containing *pedA* gene, has to be cloned into a vector to create a plasmid library. This library will contain all the plasmid fragments. An identification of clone(s) containing *pedA* gene will then be possible. pZErO™-2 vector (Invitrogen) has been chosen for cloning strategy because it has the advantage of containing resistance to kanamycin and has very high cloning efficiencies (~95%). It also allows direct selection of positive recombinants via disruption of a lethal gene called *ccd*B; insertion of a desired gene in the Multiple Cloning Site permits to disrupt expression of this *ccd*B gene. It has already been realized for this diploma work, pZErO-2 vector has been received with an insert in its MCS region, as shown on Figure 9. This made possible cultivation of transformed cells, containing pZErO-2, to extract and purify this vector. This means screening using *ccdB* gene function is no more possible with this recombinant pZErO-2 plasmid.

The size of this vector is 3297 bp with a Multiple Cloing Site situated between bases 269 and 381. Kanamycin resistance gene is situated between bases 2116 and 1322; it is derived from the bacterial transposon Tn5. pUC origin, located between bases 2502-3175, allows high copy replication and maintenance of the plasmid in *E.coli* bacteria.



* The two *Nsi* I sites in the MCS are the only sites in the vector.

† There are two tandem *Apo* I sites at this location. *Apo* I also recognizes the *Eco*R I site.

Figure 9: *pZErO™-2 vector map with its insert.*

In order to digest this vector and insert fragments from *P.damnosus* plasmid containing *pedA* gene, two restriction enzymes were chosen to create two different plasmid libraries. These enzymes had to cut pZErO-2 vector a unique time and had to be absent from *pedA* known gene. The recognition sites of these enzymes are shown on Table 4. These enzymes digested the desired gene to give cohesive ends.

Table 4: Recognition sites of the enzymes chosen for cloning strategy into pZErO-2

***Eco*RI recognition site**	***Hind*III recognition site**
5′...G▼AATTC...3′ 3′...CTTAA▲G...5′	5′...A▼AGCTT...3′ 3′...TTCGA▲A...5′

Bioinformatic tool pDRAW32 (Acoclone) was used to verify that these two enzymes cut pZErO-2 vector a unique time. The result is illustrated on Figure 10. The same analysis has been performed for *pedA* restriction with *pedA* gene, no *Eco*RI or *Hind*III recognition sites have been found using pDRAW32 (data not shown).

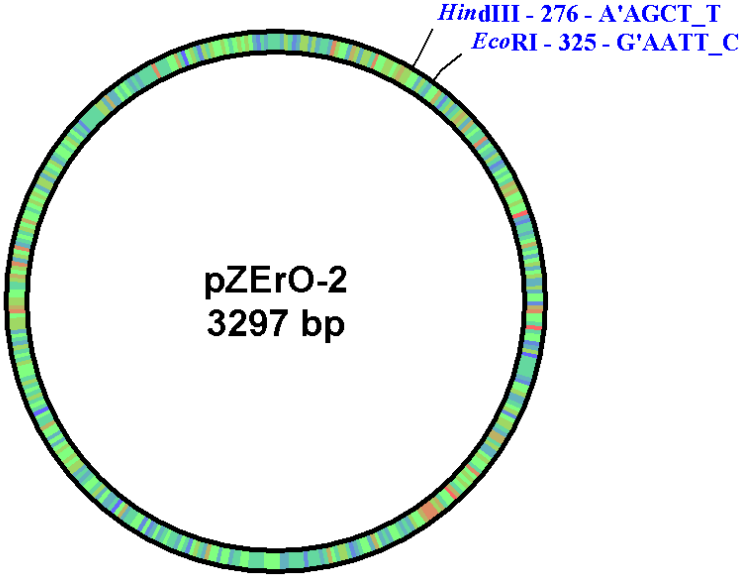


Figure 10: *pZErO-2 vector with HindIII and EcoRI recognition sites(pDRAW32).*

P.damnosus plasmid, containing *pedA* gene, is thus digested with *Eco*RI and *Hind*III to give different fragments with cohesive ends. These fragments are then ligated in pZErO-2 vector, beforehanded linearized with respectively *Eco*RI and *Hind*III and dephosphorylated. The exact amount of insert needed to obtain an optimal ligation with pZErO-2 vector is given by Invitrogen: a molecular ratio of 2:1 insert:vector, specific to cohesive ends. The amount of insert can thus be calculated with Invitrogen formula, see Equation 1, for 10 ng of linearized pZErO-2 vector of 3297 bp.

$$\text{x ng insert} = \frac{(2)\,(\text{bp insert})\,(10 \text{ ng linearized pZErO}^{\text{™}}\text{-2})}{(3297 \text{ bp pZErO}^{\text{™}}\text{-2})}$$

Equation 1: *Invitrogen formula to calculate amount of insert needed for ligation with pZErO-2 vector.*

Based on the calculation above and on the concentration of insert determined by OD_{260nm}, the volumes needed for the ligation reaction with pZErO-2 vector can be found. (Invitrogen, 2002).

1.3.5 Creation of a plasmid library

Insertion of all fragments from specific *P.damnosus* plasmid, containing *pedA* gene, into pZErO-2 vector allows to form recombinant plasmids. These plasmids are then transformed into *E.coli* TOP10 cells to form a plasmid bank. A plasmid library contains a random collection of DNA fragments from one plasmid cloned into cells of a host organism using a bacterial vector, as illustrated on Figure 11.

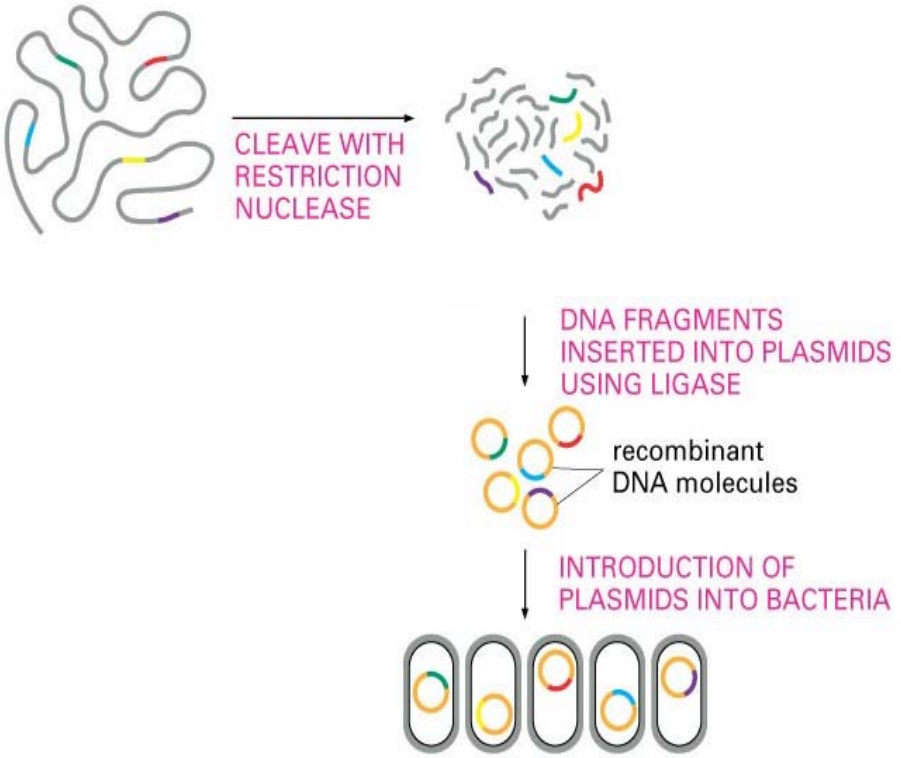


Figure 11: *Genomic library illustration (Alberts et al., 2000)*

To simplify the illustration, Figure 11 shows cloning of just a few representative fragments (*colored*). In reality, all the *gray* DNA fragments are also cloned to obtain a full genomic library.

In order to obtain a reliable plasmid library, it is crucial that the probability of containing a complete copy of any particular gene studied must be the highest possible. To do so, Clarke and Carbon's formula (1976) is used to estimate the clone number. This method based on estimated insert size will generally be preferred because of its precision and cost effectiveness relative to other methods (Gardener, 1999). For this diploma work, the number of clones is calculated with Equation 2.

$$N = \frac{\ln(1-P)}{\ln\left(1-\frac{1}{n}\right)}$$

Equation 2: *Clark and Carbon's formula*

P = Probability
n = Ratio between the genome size and the cloned fragments
N = Number of clones

1.3.6 PCR screening

Once the plasmid library is created, the transformed cell containing the recombinant plasmid with *pedA* gene has to be found in order to sequence its insert. To do so, PCR screening is one of the technique used in this diploma work; it allows to detect *pedA* gene by a *pedA* PCR-amplification.

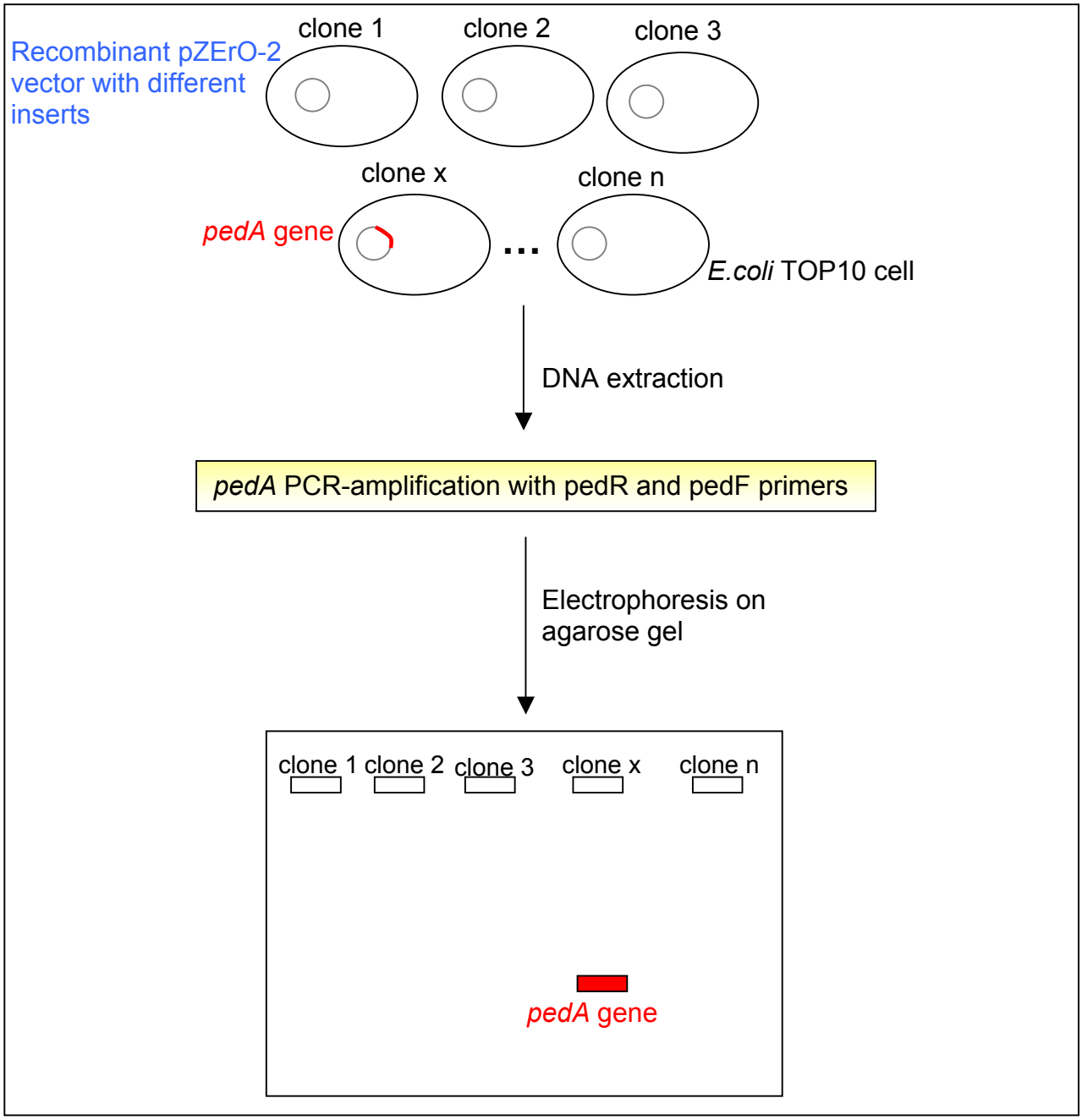


Figure 12: *Illustration of pedA PCR screening with n clones.*

PCR screening used for this diploma work is illustrated on Figure 12. DNA is extracted from transformed *E.coli* TOP10 cells and analysed via *pedA* PCR-amplification. After electrophoresis, PCR products are visible on an agarose gel and *pedA* gene is detectable; the insert of this *pedA* positive plasmid can then be sequenced to discover part of PD-1 operon.

1.3.7 Dot Blot and hybridization with pedA probe

Dot blotting is a simple technique for immobilizing bulk unfractionned DNA on a membrane in order to perform hybridization analysis, which makes possible the determination of the relative abundance of the target sequence in the blotted DNA preparations. The technique offers significant savings in time, as chromatography and complex blotting procedure for the electrophoresis gel are not required. It is thus possible to perform a screening of bacterial DNA from 96 recombinants at a time using Dot Blot 96 system (Biometra). However, Dot blot can only confirm the presence or absence of a biomolecule or biomolecules, which can be detected by the probes or the antibody. As, for this diploma work, DNA from a lot of different clones has to be analysed in order to find which clones possess the *pedA* gene in one of their plasmids, the multiplicity of applications makes the dot blot a very valuable device as standardized and reproducible assays of punctuate samples is desired.

Figure 13: *Dot Blot 96 system (Biometra) used to transfer clones DNA onto a membrane.*

Once Dot blotting and *pedA* hybridization are performed, detection with CDP-Star and chemiluminescence exposure is applied to the membrane to determine which samples contain *pedA* gene. A black dot is then visible on the membrane for every positive sample to *pedA* hybridization, as illustrated on Figure 14 in positions C4/D4 and E9/F9 (samples analyzed twice).

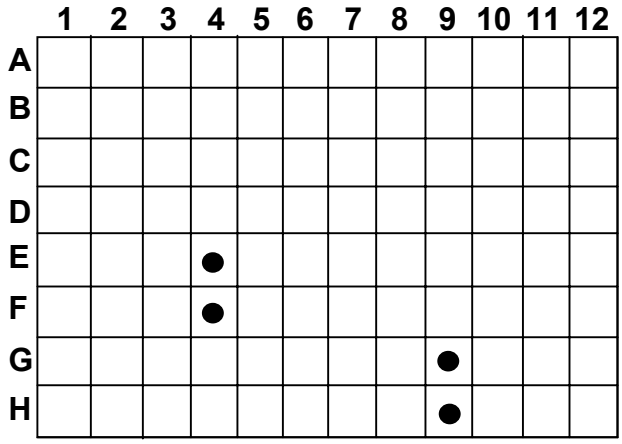


Figure 14: *Illustration of Dot blotting results with 48 clones (samples analyzed twice).*

Dot blotting combined with *pedA* hybridization allows to determine which plasmids contain *pedA* gene; their insert can then be sequenced to discover part of PD-1 operon.

1.3.8 Creation of a genetic map for pedA positive plasmids

If *pedA* positive plasmids have been found either with PCR screening or with Dot Blot/hybridization technique, a genetic map of the plasmid can be built. To do so, plasmid DNA is extracted from transformed cells and digested with different restriction enzymes. Then a comparison between theoretical pZErO-2 digestion and recombinant plasmid digestion allows to construct the genetic map of this plasmid. The theoretical genetic map of *pedA* positive recombinant plasmid is illustrated on Figure 15. The length of the insert can be longer or shorter, it depends on where restriction enzyme cuts *P.damnosus* plasmid during cloning strategy.

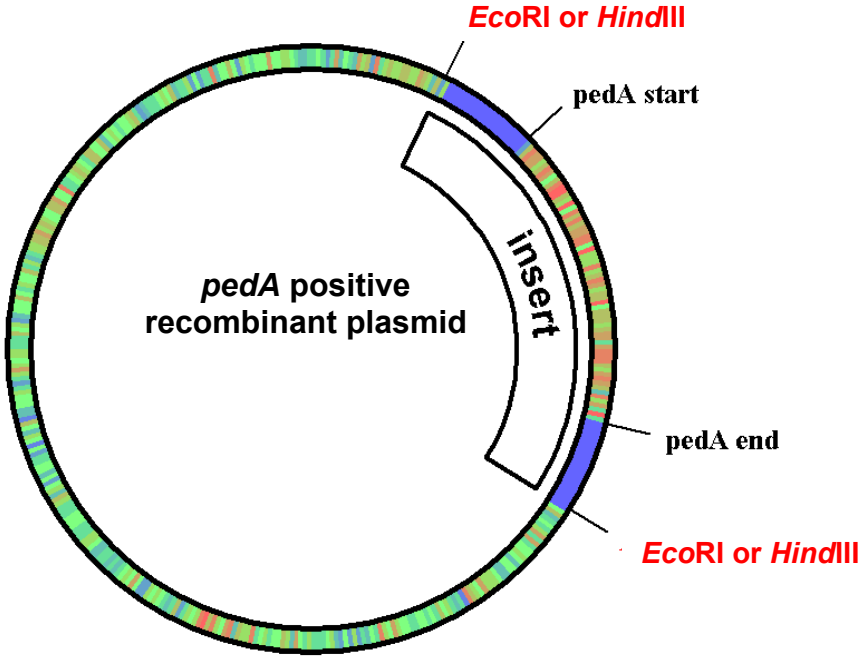


Figure 15: *Theoretical pedA positive recombinant plasmid constructed with Eco*RI or *Hind*III*; unknown sequences in blue.*

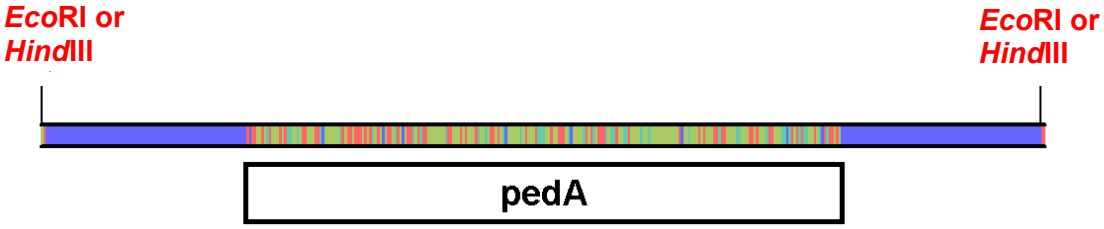


Figure 16: *Details of theoretical insert part of pedA positive recombinant plasmid, unknown sequences in blue.*

Finally, *pedA* positive plasmid(s) can be sent to an appropriate service to sequence its insert. As shown on Figure 16, insert should be constituted of *pedA* gene surrounded on both sides by unknown sequence of variable length. The goal is to sequence these blue parts shown on Figure 16, to complete operon PD-1 using an appropriate extern DNA sequencing service. For this diploma work, DNA Sequencing Services from Microsynth has been chosen. With Primer Walking single-stranded and non-assembled technique, it is possible to have sequenced the desired insert with high-speed results (Microsynth, 2007). Afterwards, when the different sequences are received, they have to be assembled. Then, *pedA* gene can be localized and, if the insert contains more than just *pedA* gene, known part of pediocin PD-1operon can be expanded. This technique can then be used again until all PD-1 operon is known.

2 Materials and Methods

2.1 Materials

Bacteria culture

MRS Broth with Tween 80	Biolofe (Italy), 4017292
DL-Malic Acid	Fluka (Switzerland), 02310
Pediococcus damnosus DSM 20331	HES-SO//Valais (Switzerland), Master Cell Bank

Pulsed-Field Gel Electrophoresis

EDTA	Fluka (Germany), 03610
Lysozyme	AppliChem (Germany), A49720010
Pronase	Roche (USA), 165921
N-Lauroylsarcosine	Sigma (USA), L-9150
Boric Acid	Sigma (Germany), B6768
Tris(hydroxymethyl)aminomethane	Acros Organics (USA), 327360010
Ethidium bromide	Sigma (Germany), E-1510
Low melt Agarose	Bio-Rad (USA), 125015A
Pulsed-field Certified Agarose	Bio-Rad (USA), 133742B
Plug Mold	Bio-Rad (USA), 1703706
Lambda Ladder	Bio-Rad (USA), 170-3635
CHEF-DR III Power Module	Bio-Rad (USA), 962BR
CHEF-Mapper^{TM} Electrophoresis cell	Bio-Rad (USA), 800BR
Variable Speed Pump	Bio-Rad (USA), 260BR
Model 1000 Mini Chiller	Bio-Rad (USA), 806BR

DNA extraction

Kit NucleoSpin® Tissue	Macherey-Nagel (France), 740952.50
Proteinase K	Qiagen (Germany), 19133
Lysosyme	Applichen (Germany), A49720010
EDTA	Fluka (Germany), 03620
Tris HCl	Applichen (Germany), A34520500

Agarose gel electrophoresis

SeAkem® LE Agarose	FMC BioProducts (USA), 50003
Ethidium bromide	Sigma-Aldrich (USA), E-1510
Boric acid	Sigma-Aldrich (USA), B6768
Tris base	Acros Organics (USA), 327360010
EDTA	Fluka (Germany), 03620
1 kb ladder, 0.5 µg/ml, Gene Ruler^{TM}	Fermentas (Germany), SM0241

Bands: 10'000-8'000-6'000-5'000-4'000-3'500-3'000-2'500-2'000-1'500-1'000-750-500-250 bp

pedA PCR-amplification

BSA 20 µg/ml	Roche (Germany), 711454
$MgCl_2$ 25 mM	Qiagen (Germany), 115564652
DNTPs 4 mM	Eppendorf (Germany)
AmpliTaq Gold^{TM} 5U/µl	Applied Biosystems (USA), G15094
PCR Gold Buffer 10x	Applied Biosystems (USA), H02115

Primer ped F	Microsynth AG (Switzerland), 333
Primer ped R	Microsynth AG (Switzerland), 334
NucleoSpin Extract II	Macherey-Nagel (France), 740609
***pedA* probe preparation**	
AlkPhos Direct Labelling Kit	Amersham (United Kingdom), RPN3680
Southern Blot	
Membrane Hybond-N^+	Amersham Biosciences (England), RPN203B
Filter Paper Electrode Wicks 104x	Pharmacia LKB (Sweden), 80-1129-52
NaOH	AppliChem GmbH (Germany), A46611000
NaCl	Fluka (Germany), 71690
Na_3Citrate dihydrate	AppliChem GmbH (Germany, A45220500
Tris Base	Acros Organics (USA), 327360010
Hybridization	
Hybridization buffer	Amersham Biosciences (England), NIF1550
Blocking reagent	Amersham Biosciences (England), NIP552
NaCl	Fluka (Germany), 71690
Hybridization oven	ShelLab MFG. Inc (USA), 1004
Bio-Link Crosslinker	Vilber Lourmat (France), BLX-Z254
Chemiluminescent signal generation	
CDP-*Star* Detection reagent	Amersham Biosciences (England), RPN3682
Plasmids digestion and ligation	
*Eco*RI, 20 U/µl	BioLabs Inc. (USA), R0101S
*Hind*III, 10 U/µl	Boehringer (Germany), 84783024
pZErO-2™ ~100 µg/ml	Lonza (Switzerland), Miniprep
Antarctic Phosphatase	BioLabs Inc. (USA), M0289S
T4 DNA ligase	BioLabs Inc. (USA), M0202S
Electroporation	
E.coli TOP10	HES-SO//Valais (Switzerland), Master Cell Bank
F- mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG	
Electroporation Cuvette Plus™	BTX® Harvard Apparatus (USA), 610
MicroPulser™	Bio-Rad (USA)
Dot Blot	
Dot Blot 96	Biometra (Germany), 053-401
EDTA	Fluka (Germany), 03620
NaOH	AppliChem GmbH (Germany), A46611000
Genetic map creation	
*Ase*I, 10 U/µl	BioLabs Inc. (USA), R0526S
*Rsa*I, 10 U/µl	Promega (USA), R6371
*Sap*I, 20 U/µl	BioLabs Inc. (USA), R0569L
*Sph*I, 5 U/µl	BioLabs Inc. (USA), R0182S

Medium

Low Salt LB Agar Plates with Kanamycin	1% Tryptone 0.5% Yeast Extract 0.5% NaCl 1.5% Agar adjust to pH 7.5 Kanamycin 50 µg/ml IPTG 1mM
Low Salt LB Medium with Kanamycin	1% Tryptone 0.5% Yeast Extract 0.5% NaCl adjust to pH 7.5 Kanamycin 50 µg/ml IPTG 1mM
SOC Medium	20 mM glucose
Agar Bacteriogical	Bioloife (Italy), 4110303
Tryptic digest of casein	Biolife (Italy), 412340
Yeast Extract	Biolife (Italy), 12220
Kanamycinsulfat	AppliChem GmbH (Germany), A1493,0005
IPTG	AppliChem GmbH (Germany), A1008,0005
NaCl	Fluka (Germany), 71690

Plasmid libraries conservation

Microtubes 2ml, PP	Sarstedt (Deutschland), 72.694.006
Glycerol anhydrous	AppliChem (Deutschland), A1123.2500

Laboratory instruments

Thermocycler Robocycler Gradient 40	Stratagene (USA)
Thermomixer Comfort 1.5 ml	Eppendorf (Germany)
Centrifuge Mikro 200	Hettich Laborapparate(Germany), 2400
Centrifuge Universal 32	Hettich Laborapparate(Germany), 1610
Uvette 50 µl	Eppendorf (Germany), 1141

2.2 Methods

2.2.1 Bacterial strains and growth conditions

Pediococcus damnosus DSM20331 is grown in MRS medium containing 3 g/L of malic acid in order to adjust pH at 5.2. Incubation is realized with 50 µl of *Pediococcus damnsosus* strain from the Master Cell Bank (HES-SO//Valais-Wallis) into 10 ml of MRS broth pH 5.2. Growth conditions are 7 days at 30°C with 150 rpm.

2.2.2 P.damnosus genomic DNA extraction

Genomic DNA is extracted from a *Pediococcus damnosus* culture using NucleoSpin® Tissue kit (Macherey-Nagel, 2006). The protocol for "Support protocol for bacteria" is followed as described below.

- 1 ml of *Pediococcus damnosus* culture is used for each extraction.
- Centrifuge for 5 min at 8'000 x g, remove supernatant.
- *Pediococcus damnosus*, being a Gram-positive bacteria, a pre-lysis has to be done with 900 µl of a solution 20 mM Tris/Cl, 2 mM EDTA, 1% Triton X-100, 20 mg/ml lysozyme at pH 8. Incubate at 37°C during 45 minutes (500 rpm). Add 25 µl of Proteinase K and incubate at 56°C during 1h15 (500 rpm).
- Vortex the sample, add 200 µl of buffer B3, vortex vigorously.
- Incubate at 70°C for 10 min, vortex briefly.
- Add 210 µl of ethanol 99%, vortex vigorously.
- Place a NucleoSpin Tissue column into a 2 ml tube, apply the sample to the column.
- Centrifuge for 1 min at 11'000 x g.
- Discard flow-through and place the column back into the tube.
- Add 500 µl of buffer BW.
- Centrifuge for 1 min at 11'000 x g.
- Discard flow-through and place the column back into the tube.
- Add 600 µl of buffer B5
- Centrifuge for 1 min at 11'000 x g.
- Discard flow-through and place the column back into the tube.
- Centrifuge for 1 min at 11'000 x g to remove residual ethanol.
- Place the column into a clean 1.5 ml tube.
- Add 100 µl of buffer prewarmed BE at 70°C
- Incubate at room temperature for 1 min in order to increase the yield of eluted DNA.
- Centrifuge for 1 min at 11'000 x g.
- Throw away the column and store the tube with DNA eluted at –20°C.

2.2.3 P.damnosus plasmid DNA extraction

Plasmid DNA is extracted from a *Pediococcus damnosus* culture using Wizard Plus SV Minipreps DNA purification system (Promega, 2006). The protocol for "Plasmid DNA isolation and purification protocols" is followed as described below.

- 1 ml of Pediococcus damnosus culture is used for each extraction.
- Centrifuge for 5 min at 10'000 x g, remove supernatant.
- Resuspend into250 µl of Cell Resuspension Solution.

- Add 250 µl of Cell Lysis Solution and mix by inverting 4 times, incubate until the cell solution clears (5 min).
- Add 10 µl of Alkaline Protease Solution and mix by inverting the tube 4 times.
- Incubate 5 min at room temperature.
- Add 350 µl of Neutralization Solution and mix by inverting the tubes 4 times.
- Centrifuge at 14'000 x g for 10 min.
- Transfer the cleared lysate to the prepared Spin column.
- Centrifuge at 14'000 x g for 1 min, remove the column from the tube and discard the flowthrough.
- Add 750 Centrifuge at 14'000 x g for 1 min of Column Wash Solution.
- Centrifuge at 14'000 x g for 1 min, remove the column from the tube and discard the flowthrough.
- Repeat the wash procedure using 250 µl of Column Wash Solution.
- Centrifuge at 14'000 x g for 2 min.
- Transfer the Spin column to a sterile 1.5ml microcentrifuge tube.
- Elute the plasmid DNA by adding 100 µl of Nuclease-Free Water to the column.
- Centrifuge at 14'000 x g for 1 min.
- Discard the Spin column and add 11 µl of 10x TE buffer.
- Store the tube with DNA eluted at –20°C.

2.2.4 Pulsed-Field Gel Electrophoresis

The protocol "Preparation of high molecular weight DNA for *Pediococcus* (and others Gram positive) and macrorestriction" (Walhen, 2003) is followed as described below.

DNA plugs preparation

- Centrifuge 2 min at 12'000 rpm, remove the supernatant.
- Add 1 ml of TE pH8, mix and centrifuge 2 min at 12'000 rpm, remove the supernatant.
- Resuspend into 200 µl of T100E pH7.5.
- Mix the cellular suspension with 400 µl of 1.5% (w/v) low melt agarose in 0.125M EDTA pH7.6 equilibrated at 50°C, mix.
- Rapidly dispense it into the plug molds and let solidify at 4°C for 15 minutes.
- Push 2 plugs into a 2ml tube and add 1 ml of T100E pH7.5 containing 10 mg/ml of lysozyme.
- Incubate 7 hours at 37°C without agitation.
- Eliminate the lysozyme solution and replace it by 1 ml of T100E containing 1.5% of laurylsarcosine and 2 mg/ml of pronase.
- Incubate one night at 37°C without agitation.
- Eliminate the pronase solution and add 700 µl of TE pH8, let 5 minutes at ambient temperature and eliminate.
- Add 650 µl of TE pH8, let 45 minutes at 37°C and eliminate.
- Add 650 µl of TE pH8, let 45 minutes at 37°C and eliminate.
- Add 600 µl of TE pH8, put the plugs in new tubes, let 45 minutes at room temperature.
- Add 500 µl of T100E pH8; plugs can be stocked in this solution for up to 3 months at 4°C.

Casting the gel

- Prepare the gel chamber with a 10-well comb as illustrated on Figure 17.
- Make a 1% agarose gel by combining 1 g of pulsed-field grade agarose with 99 ml of 0.5x TBE, and heating until completely clear of unmelted material.
- Pour the gel when the solution is cooled to 60°C.
- Allow the gel to solidify for 30 min at room temperature.
- Carefully remover the comb.
- Stock it at 4°C.

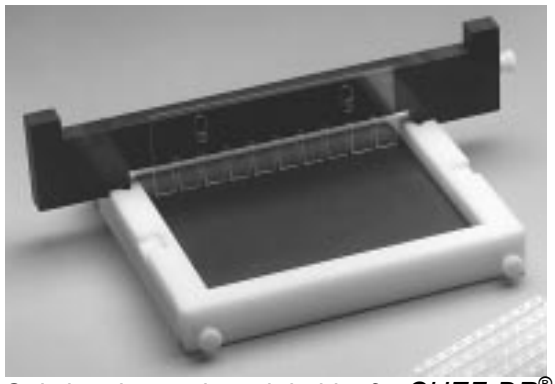


Figure 17: *Gel chamber and comb holder for CHEF-DR® III system*

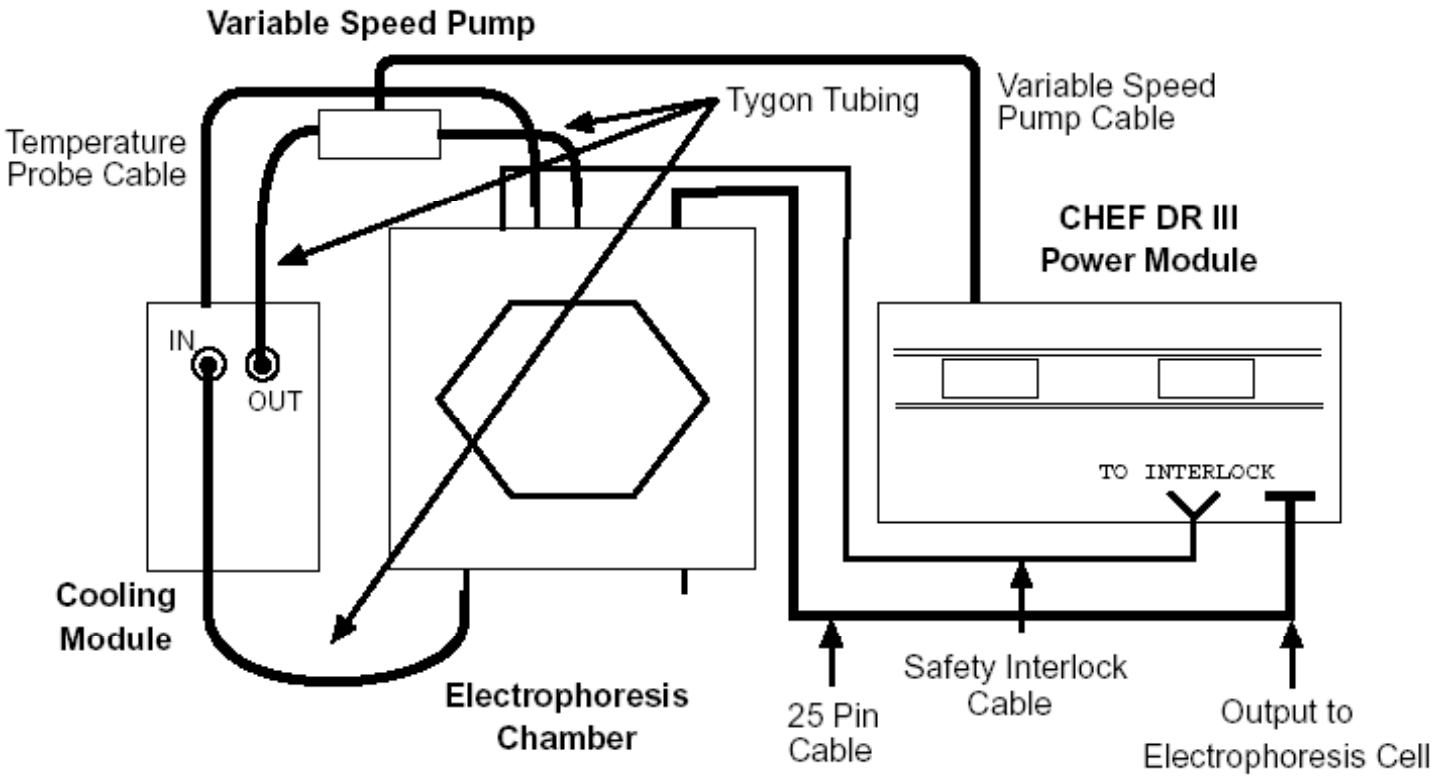
Loading the samples

- Cut size of DNA plug using a razor blade, samples should be less than 90% of the height of the wells.
- Place agarose plugs onto the front walls of the wells using a spatula, gently press them to the bottom of the wells.
- Place DNA size standards Lambda into wells at each sides of the gel.
- Fill each well with 1.5 % (w/v) LM agarose.
- Allow the agarose to harden at room temperature for 15 minutes.

CHEF-DR® III system assembling

The CHEF-DR® III system has to be assembled following the protocol “CHEF-DR III Pulsed-Field Electrophoresis Systems, Instruction Manual and Application Guides” (Bio-Rad, 1999) as illustrated on Figure 18.

- The Power Module contains all the electronics needed to perform pulsed-field electrophoresis.
- The Electrophoresis Cell consists of an acrylic box with 24 horizontal electrodes arranged in a hexagon. It has to be levelled with the four levelling feet at each corner.
- The Variable Speed Pump helps providing a suitable flow rate of buffer through the chamber.
- The Model 1000 Mini Chiller, also called Cooling Module, is a portable refrigerated apparatus.



Electrophoresis

- Fill the electrophoresis chamber with 2 liters of 0.5x TBE.
- Turn on pump and chiller, set pump to 70 (1 L/min) and temperature to 14 ºC.
- Allow temperature of buffer to equilibrate for 30 min.
- Place the gel with samples charged into the electrophoresis chamber and let the temperature equilibrate for 15 minutes.
- Check the buffer level to insure that the gel is covered by about 2 mm of buffer.
- Set the PFGE conditions as below:
  - Linear pulse time: 10 s to 100s
  - Angle: 120°
  - Temperature: 14°C
  - Voltage: 6 V/cm
  - Run time: 18 h
- Press PAUSE/START RUN.

Removing and staining the gel

- Remove the gel form the cell.
- Put the gel into EDTA 0.125M pH7.6 containing 1 µg of ethidium bromide per ml, agitate for 20 min.
- Put the gel into TBE 0.5x, agitate for 20 min.
- Put the gel into TBE 0.5x, agitate for 20 min.
- Take a picture of the gel under UV (GelDoc 1000).

2.2.5 Extraction of plasmids form agarose gel

NucleoSpin Extract II kit (Macherey-Nagel)

- Excise DNA fragment
- For each 100 mg of agarose gel add 200 µl Buffer NT.
- Incubate sample at 50°C until the gel slices are dissolved (5-10 min).
- Place a NucleoSpin® Extract II Column into a Collection load sample.
- Add 600 µl Buffer NT3.
- Centrifuge for 1 min at 11'000 x g. Discard flow-through and place the column back into the Collection Tube.
- Centrifuge for 2 min at 11'000 x g to remove Buffer NT3 quantitatively. Discard flow-through and place the column back into the Collection Tube.
- Place the column into a clean 1.5 ml microcentrifuge tube.
- Add 15-50 µl of NE Elution Buffer NE and incubate at room temperature for 1 min to increase the yield of eluted DNA.
- Centrifuge for 1 min at 11,000 x g.
- Throw away the column and store the tube with DNA eluted at –20°C.

NuceloTrap kit (Macherey-Nagel)

- Excise DNA fragment
- For each 100 mg agarose gel add 300 µl NT1.
- Vortex the NucleoTrap® Suspension thoroughly, resulting in a homogeneous mixture. For each µg of DNA add 4 µl of the NucleoTrap® Suspension, but at least 10 µl.
- Incubate sample at 50°C until the gel slices are dissolved (5-10 min).
- Add 500 µl buffer NT2 to the pelleted silica matrix and vortex briefly for resuspension of the pellet. Centrifuge for 30 s at 10'000 x g and remove the supernatant completely.
- Add 500 µl buffer NT3 and vortex briefly. Centrifuge for 30 s at 10'000 x g and remove the supernatant completely.
- Add 500 µl buffer NT3 and vortex briefly. Centrifuge for 30 s at 10'000 x g and remove residual buffer NT3 completely.
- Dry the pelleted silica matrix at room temperature or at 37°C for 10-15 min.
- Add 25-50 µl elution buffer NE to the silica matrix. Resuspend the pellet by vortexing.
- Incubate the mixture at room temperature for 15 min.
- Centrifuge the sample for 30 s at 10'000 x g and transfer the DNA containing supernatant to a clean tube.
- Throw away the column and store the tube with DNA eluted at –20°C.

JetSorb kit (Genomed)

- Excise DNA fragment
- For each 100 mg gel slice add 300 µl buffer A1 and 10µl JETSORB suspension.
- Incubate sample at 50°C until the gel slices are dissolved (5-10 min).
- Centrifuge for 30 s at 10'000 x g, remove the supernatant completely.
- Resuspend the pellet with 300 µl of buffer A1.
- Centrifuge for 30 s at 10'000 x g, remove the supernatant completely
- Resuspend the pellet with 300 µl of buffer A2.

- Centrifuge for 30 s at 10'000 x g, remove the supernatant completely
- Resuspend the pellet with 300 µl of buffer A2.
- Add 20 µl TE buffer
- Incubate at 50°C for 5 min to increase the yield of eluted DNA.
- Centrifuge for 30 sec at 11,000 x g.
- Throw away the column and store the tube with DNA eluted at –20°C.

Restriction in agarose plugs and NuceloSpin Extract II kit (Machery-Nagel)

- Excise DNA fragment
- Add 500 µl of TE 0.1 pH 7, incubate 2 hours at room temperature.
- Remove TE 0.1 and repeat (Add 500 µl of TE 0.1 pH 7, incubate 2 hours at room temperature).
- Remove TE 0.1.
- Add 300 µl of restriction buffer 1x and incubate 30 min at 4°C.
- Remove restriction buffer.
- Add 300 µl of restriction buffer 1x and 5 µl of restriction enzyme, incubate at 37°C overnight.
- Extracted DNA fragments following NucleoSpin Extract II kit protocol (Macherey-Nagel) as described on chapter *2.2.5 Extraction of plasmids form agarose gel.*

2.2.6 Preparation of pedA probe

The probe used in hybridization is prepared by *pedA* PCR-amplification using the protocol "Hybridization-Ribotyping" (Forré, 2003).

- Prepare the mastermix (here for one sample) as described below.

Distilled wasser	25.75 µl
BSA 20 µg/ml	5 µl
$MgCl_2$ 25 mM	4 µl
dNTPs 4 mM	2.5 µl
AmpliTaq Gold™ 5U/µl	0.25 µl
PCR Gold Buffer 10x	5 µl
Primer ped F	1.25 µl
Primer ped R	1.25 µl

- Add 5 µl of *P.damnosus* genomic DNA with 45 µl Mastermix for each sample.
- Perform *pedA* PCR-amplification with the following PCR protocol.

Initial denaturation	5 min	95°C
Denaturation	50 sec	95°C
Annealing	95 sec	55°C
Extension	115 sec	72°C
Final extension	5 min	72°C
Cycles	40	

The PCR product is extracted from the electrophoresis gel using the NucleoSpin® Extract II kit (Macherey-Nagel, 2006). The protocol for "Protocol for DNA extraction from agarose gels" was followed as described below.

- Excise DNA fragment from the agarose gel with a clean scalpel.
- Determine the weight of the gel slice and transfer it to a tube.
- Add 200 µl of buffer NT for each 100 mg of agarose gel.
- Incubate at 50°C for 10 min, vortex briefly the sample every 3 min to dissolve gel slices completely.
- Place a NuceoSpin Extract II column into a 2 ml tube and load the sample.
- Centrifuge for 1 min at 11'000 x g.
- Discard flow-through and place the column back into the tube.
- Add 600 µl of buffer NT3.
- Centrifuge for 1 min at 11'000 x g.
- Discard flow-through and place the column back into the tube.
- Centrifuge for 2 min at 11'000 x g to remove buffer NT3 quantitavely.
- Place the column into a clean 1.5 ml tube.
- Add 25 µl of NE buffer.
- Incubate at room temperature for 1 min in order to increase the yield of eluted DNA.
- Centrifuge for 1 min at 1000 x g.
- Throw away the column and store the tube with DNA eluted at –20°C.

The final step of *pedA* probe preparation is done from extracted DNA after PCR using the protocol "Hybridization-Ribotyping" (Forré, 2003) as described below.

- Dilute 20 µl of cross-linker solution with 80 µl of water supplied to give the working concentration.
- Dilute purified PCR product to a concentration of 10 ng/µl using water supplied.
- Place 10 µl of diluted DNA sample in a 1.5 ml tube, denature by heating in a boiling water bath for 5 minutes.
- Immediately cool the DNA solution on ice for 5 minutes, spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
- Add 10 µl of reaction buffer, mix thoroughly but gently.
- Add 2 µl of labelling reagent, mix thoroughly but gently.
- Add 10 µ of cross-linker working solution, mix thoroughly but gently. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
- Incubate for 30 minutes at 37°C.
- Stock the probe on ice for up to 2 hours or store it in 50% (v/v) glycerol at a temperature of –15°C for up to 6 months.

2.2.7 Southern Blot

DNA transfer via Southern blotting is done from the Pulsed-Field Electrophoresis gel using the protocol "Analysis of DNA sequences by blotting and hybridization" (Ausubel, 1999) as describe below

- Denature the samples by covering the gel in denaturation buffer for 2 x 15 min at room temperature, rince in distilled water.
- Neutralize the samples by covering the gel in neutralization buffer for 2 x 15 min at room temperature, rince in distilled water.
- Prepare the Southern Blot structure; use Figure 19 as a guide.

- Cut 4 pieces of Whatmann 3MM paper to the same size as the agarose gel, put them on a flat surface and wet them with 20x SSC.
- Place the gel on these filter papers, squeeze out air bubbles.
- Cut 4 strips of plastics wrap and place over the edges of the gel.
- Cut the Hybond-N+_nylon transfer membrane the same size as the agarose gel, wet it in a distilled water bath. Allow the membrane to submerge for 5 minutes.
- Place the wetted membrane on the surface of the gel.
- Flood the surface of the membrane with 20x SSC.
- Cut 4 pieces of Whatman 3 MM paper to the same size as the membrane, wet two of them and place them on the top of the membrane. Place the other two on top of them.
- Cut a piece of pampers to the same size as the membrane, stack it on top of the Whatman 3 MM papers.
- Lay a glass plate on top of the structure.
- Place a weight of about 1 kg on top to hold everything in place.
- Leave 1 hour.

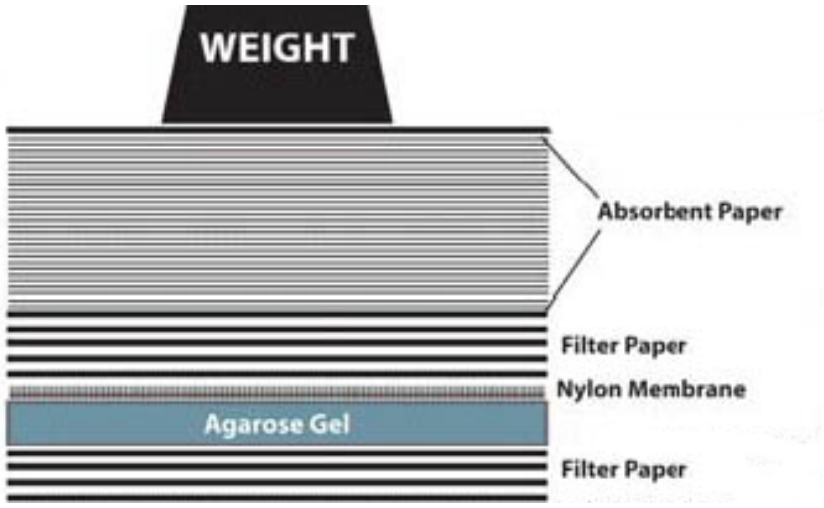


Figure 19: *Southern Blot illustration*

- Disassemble the transfer structure and recover the membrane. Mark in pencil the positions of the wells and the orientation of the membrane.
- Photograph the gel to assess transfer efficiency.
- Rinse the membrane with 2x SSC, place it on a sheet of Whatmann 3MM paper, allow to air dry.

2.2.8 Immobilization of DNA

Immobilization of DNA after Southern blotting step is done using UV irradiation at 254 nm with Bio-Link Crosslinker (Vilber Lourmat):

- Wrap the membrane with plastic wrap.
- Place it in the UV crosslinker with DNA-side-up.
- Irradiate the membrane at 254 nm by pressing START.
- Stock it at room temperature between two Whatmann 3 MM papers.

2.2.9 Hybridization

Hybridization of the membrane with *pedA* probes is realized from the protocol "Hybridization and ribotyping" (Forré, 2003) as described below.

- Prepare AlkPhos Direct hybridization buffer by adding NaCl to the hybridization buffer solution to give a concentration of 0.5 M. Add slowly blocking reagent to a final concentration of 4 % (w/v). Mix for 2 hours at room temperature.
- Heat the required volume of AlkPhos Direct hybridization buffer to 55°C for 30 minutes (0.25 ml/cm^2 of membrane).
- Place the membrane into the hybridization tube with the hybridization buffer, prehybridise at 55°C for 15 min.
- Add 10 ng of pedA probe per ml of buffer.
- Hybridise at 55°C overnight.

2.2.10 Post hybridization

- Preheat the primary wash buffer to 55°C for 30 min. This is used at a volume of 5 ml/cm^2 of membrane.
- Transfer the membrane to this solution and wash at 55°C for 10 min in the hybridization oven.
- Perform a further wash in fresh primary buffer at 55°C for 10 min in the hybridization oven.
- Wash the membrane with secondary wash buffer for 5 min at room temperature with gentle agitation.
- Wash the membrane with fresh secondary wash buffer for 5 min at room temperature.

2.2.11 Chemiluminescent signal generation & detection with CDP-STAR

Detection is done using detection reagent CPS-Star. The chemiluminescent signal is obtained with Gel Doc 1000 and Quantity One software.

- Pipette CPS-Star detection reagent on the membrane and leave for 5 min.
- Drain off excess of reagent.
- Detect light emission with Chemidoc XRS, exposure time of 20 min.

2.2.12 Cloning of plasmid fragments into pZErO-2

Cloning of *P.damnosus* desired fragments from plasmid, containing *pedA* gene, into pZErO-2 vector is realized from the protocol "Zero Background/Kan Clong Kit" (Invitrogen, 2002) as described below:

- Digest 1 µg of pZErO-2 vector in total volumes of 10 µl for 15 min using the recommended buffer, temperature and reaction conditions described by the manufacturer of the chosen restriction enzyme.
- Add Antarctic Phosphatase using the recommended buffer and incubate 15 min at 37°C.
- Digest 1 µg of *P.damnosus* plasmid DNA or extracted plasmid α and β in total volumes of 10 µl for 30 min using the recommended buffer, temperature and reaction conditions described by the manufacturer of the chosen restriction enzyme.
- Add TE buffer to the both digestion solution to a final volume of 100 µl.
- Heat the reaction to 70°C for 10 min.

- Cool to room temperature, then place on ice.
- Prepare the following ligation reaction:

Digestion vector (10 ng)	1 µl
Digested plasmid	x µl
Ligation Buffer 10x	1 µl
T4 DNA ligase (4U/µl)	0.5 µl
Total volume	10 µl

- Incubate at 16°C overnight and place vials on ice.
- Prepare LB plates containing 50 µg/ml of kanamycin and store at 4°C.
- Prepare LB medium containing 50 µg/ml of kanamycin liquid medium for DNA minipreps.
- Proceed to transformation.

2.2.13 Insertion by electroporation of recombinant plasmids into E.coli cells

- Thaw on ice the appropriate number of microcentrifuge tubes of TOP10 electrocompetent cells.
- Dilute ligation reaction with 10 µl of sterile water and place at 65°C for 5 min. This dilution reduce the salt concentration, excess salt may cause arcing during electroporation.
- Place one Eppendorf tube and one elecroporation cuvette on ice for each sample.
- Add 3 µl of the ligation reaction to each tube containing 40 µl of competent cells.
- For the control reaction, add 3 µl of pZErO-2 vector to a separate tube of 40 µl competent cells.
- Incubate all tubes on ice for 1 min.
- Transfer the cell/DNA mix to an electroporation cuvette. Place the cuvette in the chamber of the MicroPulser (Bio-Rad) and discharge an electrical pulse.
- Remove the cuvette and immediately add 450 µl of SOC medium at room temperature, transfer to an Eppendorf tube and place on ice.
- Incubate all tubes at 37°C for 60 min at 300 rpm.
- Plate 100 µl of the transformation mix onto a LB-kanamycin plate for each sample.
- Concentrate the rest of the solution by centrifugation at 14'000 rpm for 2 min and remove the supernatant. Resuspend and plate it onto another LB-kanamycin plate for each sample.
- Incubate at 37°C for 24 hours.

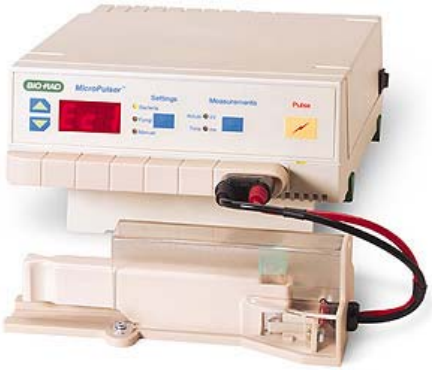


Figure 20: *MicroPulser (Bio-Rad) used for electroporation of recombinant plasmid into E.coli supercompetent cells.*

2.2.14 Clones purification

- Isolate a clone by picking one colony and place it onto a new LB-kanamycin plate.
- Incubate at 37°C for 24 hours.
- Remove plates from incubator and pick at least 10 kanamycin resistant transfomants.
- Inoculate into 2 ml of LB medium containing 50 µg/ml kanamycin.
- Grow overnight at 37°C, 400 rpm.

2.2.15 DNA extraction

DNA extraction after clone purification is done using the NucleoSpin® Tissue kit (Macherey-Nagel, 2006). The protocol for "Support protocol for bacteria" is followed as described on chapter *2.2.2 P.damnosus genomic DNA extraction.*

2.2.16 PCR screening

PCR screening is done following the protocol "Hybridization-Ribotyping" (Forré, 2003).

- Prepare the mastermix (here for one sample) as described below.

Distilled wasser	25.75 µl
BSA 20 µg/ml	5 µl
$MgCl_2$ 25 mM	4 µl
dNTPs 4 mM	2.5 µl
AmpliTaq Gold™ 5U/µl	0.25 µl
PCR Gold Buffer 10x	5 µl
Primer ped F	1.25 µl
Primer ped R	1.25 µl

- Add 5 µl of *P.damnosus* genomic DNA of each clone with 45 µl Mastermix.
- Perform *pedA* PCR-amplification with the following PCR protocol.

Initial denaturation	5 min	95°C
Denaturation	50 sec	95°C
Annealing	95 sec	55°C
Extension	115 sec	72°C
Final extension	5 min	72°C
Cycles	40	

2.2.17 Dot blot and hybridization with pedA probe

DNA transfer to a membrane using Dot blotting technique is realized from the protocol "Dot Blot 96" (Biometra, 1995) and "Dot blotting of DNA onto positively charged nylon membrane using a manifold" as described below:

- Cut a piece of positively charged nylon membrane to the appropriate size.
- Pour distelled water to a depth of 0.5 cm in a glass dish and place the membrane on the surface, allow submerging for 10 min.
- Cut a piece a piece of Whatmann 3 MM filter paper to the size of the manifold, wet in 6xSSC.
- Add NaOH 1 M and 200 mM EDTA pH 8.2 to each sample to give a final concentration of 0.4 M NaOH/10 mM EDTA.
- Heat the solution at 99°C for 10 min.

- Assemble the unit as illustrated on Figure 21.

- Prewash the membrane with 500 µl distilled water per well.
- Wash the membrane with 500 µl of 6xSSC to each well, allow the SSC to filter through.
- Spin the DNA samples in a microcentrifuge for 5 sec.
- Apply the DNA samples to the wells, allow the samples to filter through.
- Place 500 µl of 6xSSC into every empty wells.
- Rince the wells with 500 µl of NaOH 0.4 M; allow a small amount of wash solution to remain in the wells.
- Wash the membrane with 2xSSC.
- After the wash solution has flowed through the membrane, maintain the vacuum for an additional 5 min to dry the membrane.
- Remove the membrane from the Dot Blot 96 apparatus and allow it to dry completely at room temperature.

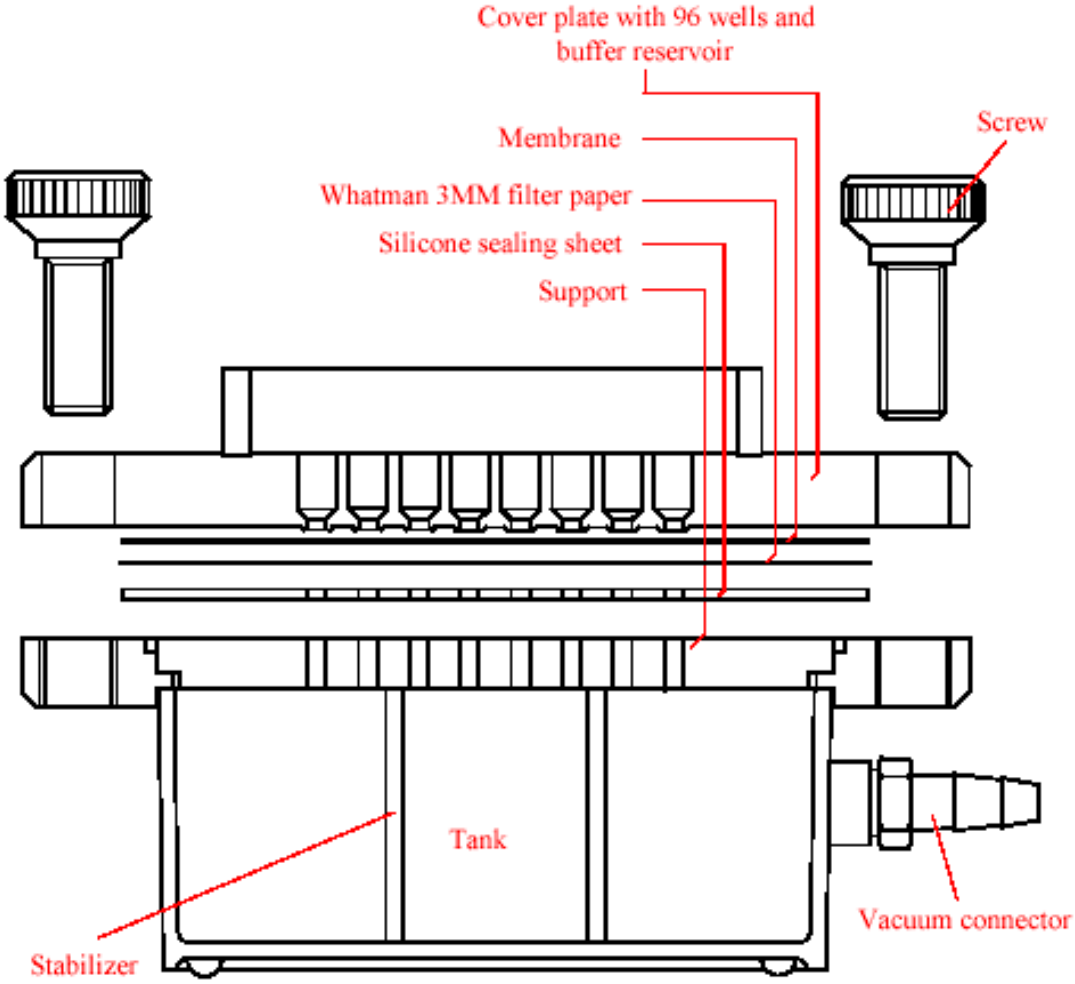


Figure 21: *Set up of Dot Blot 96 (Biometra®) sandwich*

The further treatment of the membrane,in order to perform hybridization with *pedA* probe is identical to chapters *2.2.6. Immobilisation of DNA, 2.2.7. Hybridization, 2.2.8. Post hybridization* and *2.2.9 Chemiluminescent signal generation & detection with CDP-STAR*.

2.2.18 Creation of genetic map for pedA positive recombinant plasmids

Plasmid DNA is extracted from *pedA* positive clones in LB-kanamycin with Wizard Plus SV Minipreps kit (Promega) following the protocol "Plasmid DNA isolation and purification protocols", as described on chapter *2.2.3. Plasmid DNA extraction,* before performing the restriction strategy to construct their genetic map.

- Digest 0.5 µg of extracted plasmid DNA using the recommended buffer, temperature and reaction conditions described by the manufacturer of the chosen restriction enzyme.
- Digest 0.5 µg of pZErO-2 vector using the recommended buffer, temperature and reaction conditions described by the manufacturer of the chosen restriction enzyme.
- Incubate at 37°C for 90 min.
- Prepare an 1% agarose gel and perform an electrophoresis by putting every time plasmid DNA and pZErO-2 digested with the same enzyme.
- Compare bands visible on the gel after UV exposure.
- Construct the genetic map of each recombinant plasmid.

2.2.19 Preparation of pedA positive plasmid(s) for extern DNA sequencing

Plasmid DNA is extracted from *pedA* positive clones in LB-kanamycin with Wizard Plus SV Minipreps kit (Promega) following the protocol "Plasmid DNA isolation and purification protocols", as described on chapter *2.2.3. Plasmid DNA extraction.*

- Quantify DNA solution by spectrophometry; it has to be at least 100 ng/µl.
- Note down all the essential informations on the tubes and close it with parafilm.
- Send it in a bubble letter to Microsynth DNA Sequencing Department, using Primer Walking single-stranded and non-assembled service.

2.2.20 Plasmid libraries conservation

Plasmid libraries created have to be conserved in glycerol 87% and stock at –70°C.

- Add 1ml of glycerol anhydrous in a cryotube.
- Sterilize these cryotubes at 121°C for 15 min.
- Add 1 ml of LB-kanamycin culture of each clone.
- Mix by inverting 2-3 times.
- Incubate at room temperature for 1 hour.
- Stock the cryotubes at –70°C.

3 Results

3.1 DNA extraction

Pediococcus damnosus DSM 20331 culture has been grown at 30°C 150 rpm during 7 days in MRS broth pH 8 medium. Verification under microscopy shows no contamination of the bacterial culture. DNA extraction and preparation of DNA plugs for Pulsed-Field Electrophoresis can thus be done using directly this controlled *P. damnosus* culture.

P.damnosus genomic DNA extraction has been realized using NucleoSpin® Tissue Kit (Macherey-Nagel), an electrophoresis has then been realized on a 1% agarose gel shown on Figure 22.

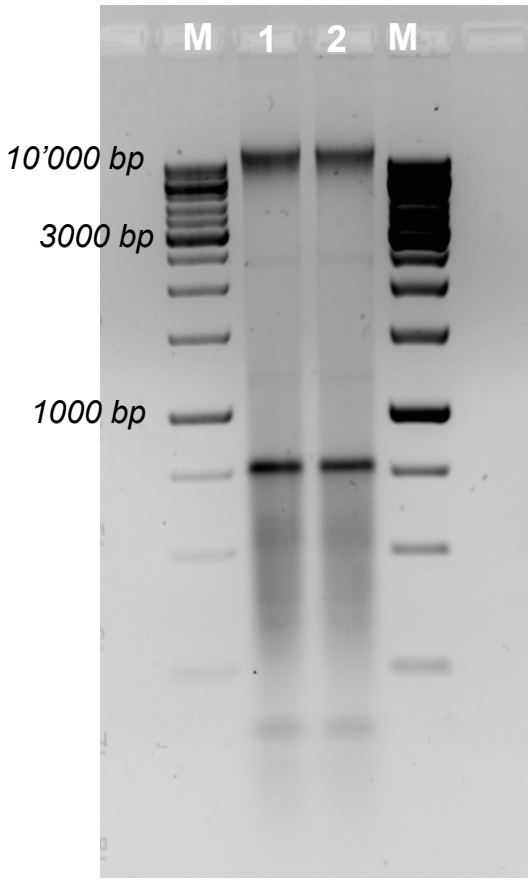


***Figure 22**: P.damnosus genomic DNA electrophoresis gel*
M: GeneRuler 1 kb DNA ladder (Fermentas), 1-2: P.damnosus genomic DNA

On Figure 22, isolated DNA from *P.damnosus* is visible on lanes 1 and 2. The first thick band, superior to 10 kbp, corresponds to chromosomal *P.damnosus* DNA. The other two smaller bands, around 800 bp and inferior to 100 bp, correspond to plasmid DNA.

Large DNAs are not well separated using classic electrophoresis. Therefore, Pulsed-Field electrophoresis has been chosen to separate all kinds of *P.damnosus* DNAs. Identification with pedA hybridization would be clearer using this method.

3.2 *pedA* probe preparation

pedA PCR-amplification was realized on *P.damnosus* genomic DNA using pedF and pedR primers. The picture taken after electrophoresis on a 1% agarose gel is shown on the left side of Figure 23. This same gel after *pedA* bands extraction is shown on the right side.

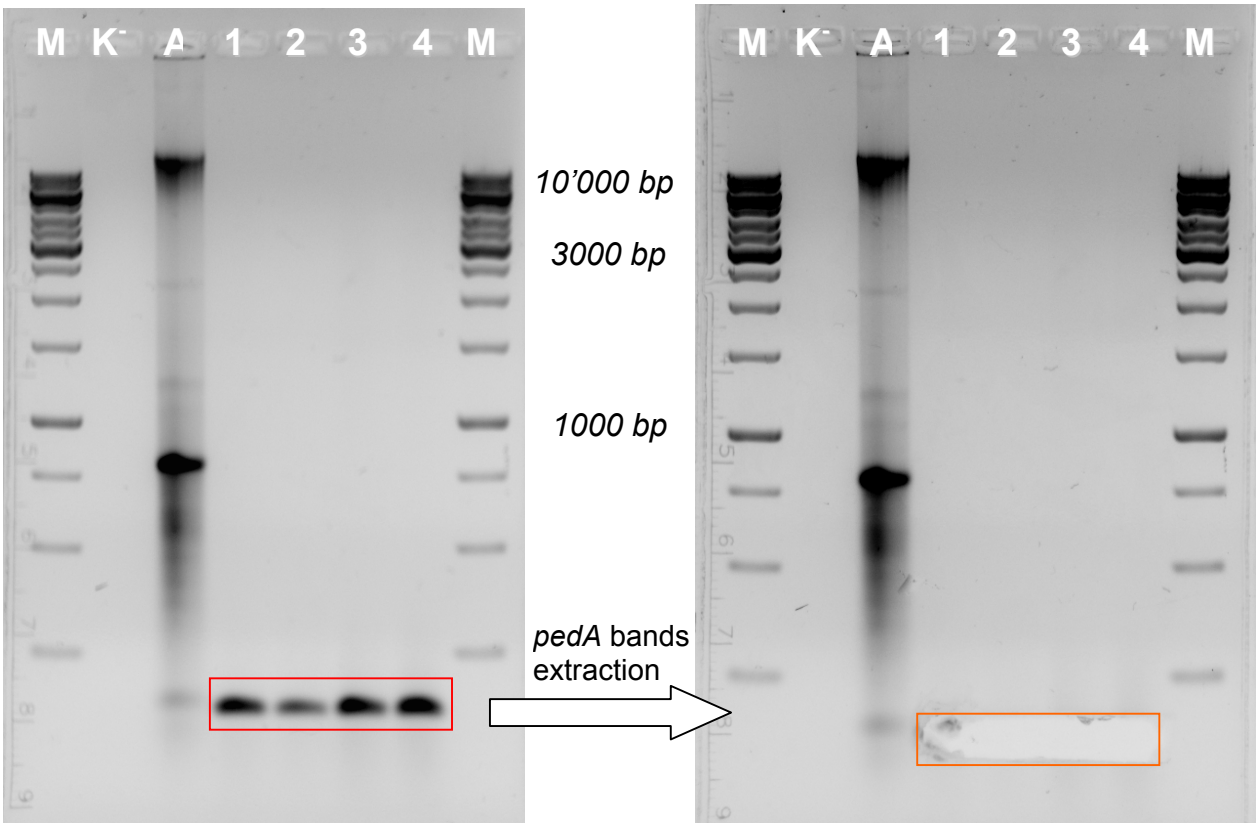


Figure 23: *pedA PCR-amplification electrophoresis gel (picture on the left) and pedA extraction (picture on the right)*
M: GeneRuler 1 kb DNA ladder (Fermentas); K⁻: Negative control; A: P.damnosus DNA; 1-4: pedA PCR-amplification.

As it is shown on Figure 23, *pedA* amplification by PCR shows a unique DNA band inferior to 250 bp for all 4 samples. As described on appendices *8.4*, PCR product using primers pedF and pedR has a theoretical size of 139 bp; it corresponds to the bands of lanes 1 to 4. Negative control, containing mastermix with water, shows no amplification after the PCR step. Concerning lane A, it reveals *P.damnosus* DNA without any amplification.
DNA bands corresponding to *pedA* gene have then been extracted from the gel using NulceoSpin Extract II kit. After purification, DNA concentration has been determined by spectrophotometry: [dsDNA] = 22.1 µg/ml

pedA probe has then been prepared using the purified PCR product with addition of the enzyme alkaline phosphatase. A control of this *pedA* probe has been performed by adding 2 µl of this probe on a Hybond H^+ membrane, fixed by UV light and detected with CDP-Star reagent. The result after chemiluminescence exposure shows a positive result for the interaction between *pedA* probe and CDP-Start substrate. Indeed, a light production is detectable and therefore, *pedA* probe can be used for hybridization of the Hybond-N^+ membrane (data not shown).

3.3 *Pulsed-Field Gel Electrophoresis*

Pulsed-Field electrophoresis of prepared plugs containing *P.damnosus* DNA is realized on a 1% pulsed-field agarose gel in TBE 0.5x during 18 hours. Linear pulse time is fixed from 10 sec to 100 sec, the angle to 120° and the voltage to 6 V/cm. Temperature of the TBE 0.5% medium is kept constant at 14°C. Figure 24 represents pulsed-field electrophoresis gel after UV detection (GelDoc 1000).

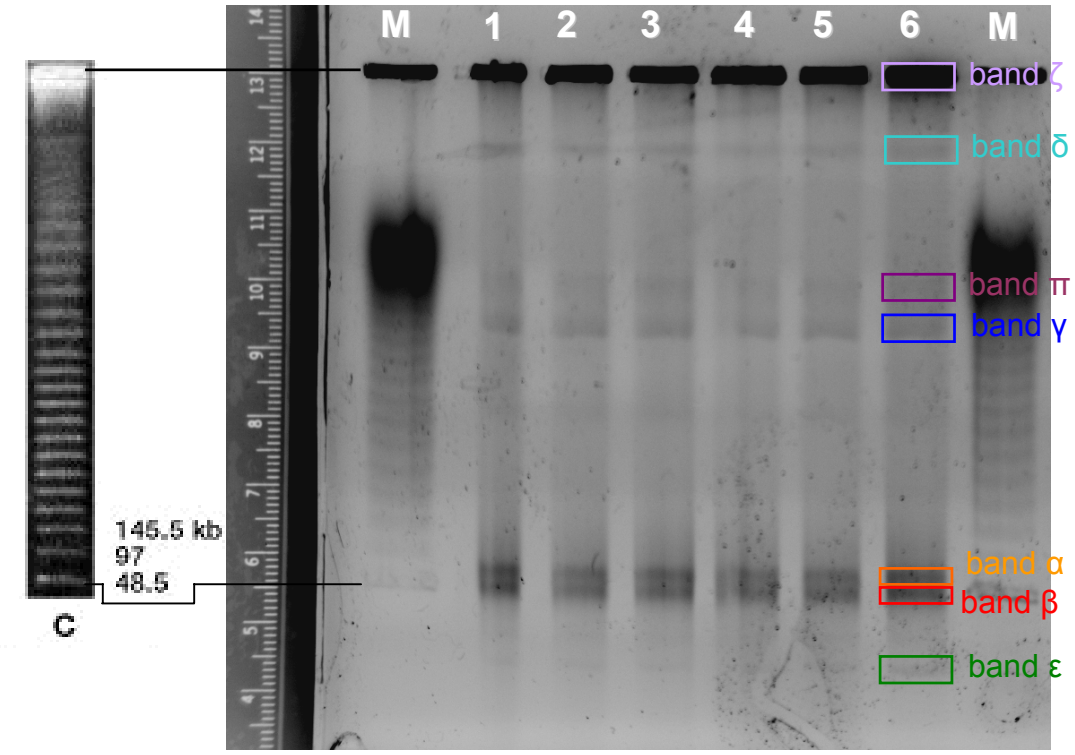


Figure 24: *Pulsed-Field electrophoresis gel of P.damnosus DNA*
M: Lambda Ladder (Bio-Rad); 1-6: P.damnosus DNA plugs

Figure 24 shows PFGE results after 18 hours of migration. Lanes 1 to 6 represent *P.damnosus* DNA plugs, a prominent part of this DNA stayed in the gel wells, corresponding to band ζ; the pulsed-field electrophoresis is therefore not optimal. However, some bands are detectable on this pulsed-field electrophoresis gel. Band δ is detectable on top of Lambda ladder; it is certainly chromosomal DNA. Two bands γ and π are visible at about 600 kbp, it could be two of *P.damnosus* plasmid or simply one of its plasmid in two different forms. Two thick bands α and β are observable at about 50 kbp and seems to correspond to one plasmid in two different forms. Finally, band ε corresponds to one of *P.damnosus* plasmid inferior to 48 .5 kbp.
Part of DNA on Figure 24, lanes 5,6 and M to be exact, has then been transferred on a Hybond-N^+ membrane by Southern blotting in order to perform hybridization with *pedA* probe. Concerning lanes 1 to 4 of Figure 24, they will be used for DNA extraction from the pulsed-field electrophoresis gel after the hybridization results have been found. Indeed, DNA bands containing *pedA* gene will be detected after hybridization and chemiluminescence detection.
Results from a previous work (Ledda, 2007) show *pedA* gene in either plasmids from bands α or β; this affirmation needs to be confirmed before going any further in this project.

3.4 Southern Blot

DNA transfer from Pulsed-Field electrophoresis gel to Hybond-N^+ membrane using Southern Blot technique has been immobilized by UV irradiation. A comparison between the pictures of the gel taken before and after DNA transfer, shown on Figure 25, proves that there is almost no more DNA on the electrophoresis gel after Southern Blot.

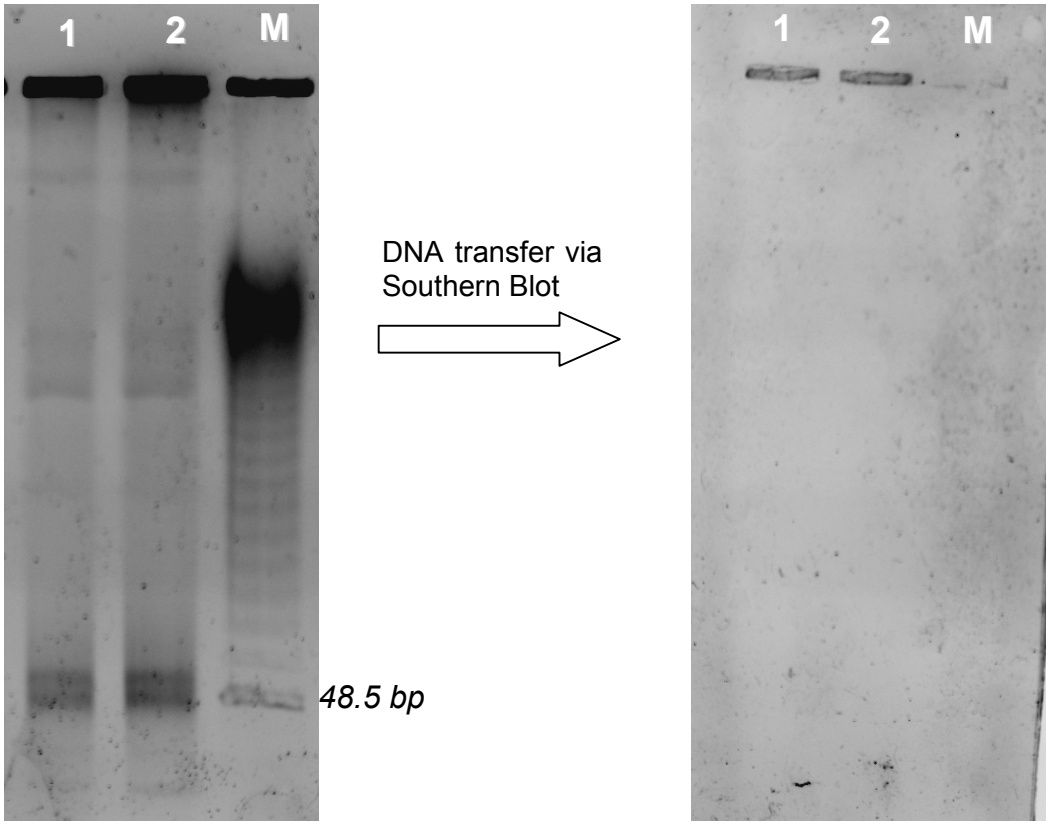


Figure 25: *Pulsed-Field electrophoresis gel before DNA transfer via southern blotting (picture on the left) and after transfer (picture on the right).*
M: Lambda Ladder (Bio-Rad); 1-2: P.damnosus DNA plugs

As shown on the left side of Figure 25, lanes 1 and 2 as well as lane M, corresponding to the Lambda ladder, are transferred via Southern Blot on the Hybond-N^+ membrane. The agarose gel after transfer by upward capillary action is then analyzed under UV to verify the efficiency of the transfer. This result is shown on the right side of Figure 25; DNA bands are no more detectable on the agarose gel. This control allows to prove that the upward capillary action did work. However, this control is not sufficient to be sure that DNA has been transferred onto the Hybond-N^+ membrane.

3.5 Hybridization with pedA *probe*

Hybridization of the Southern Blot membrane has been realized using *pedA* probe in order to detect which DNA bands contain *pedA* gene. After washing out the membrane and detection with CDP-Star solution, an analysis under chemiluminescence light has been performed during 40 minutes (GelDoc 1000). The resulting picture taken after this step is shown on Figure 26.

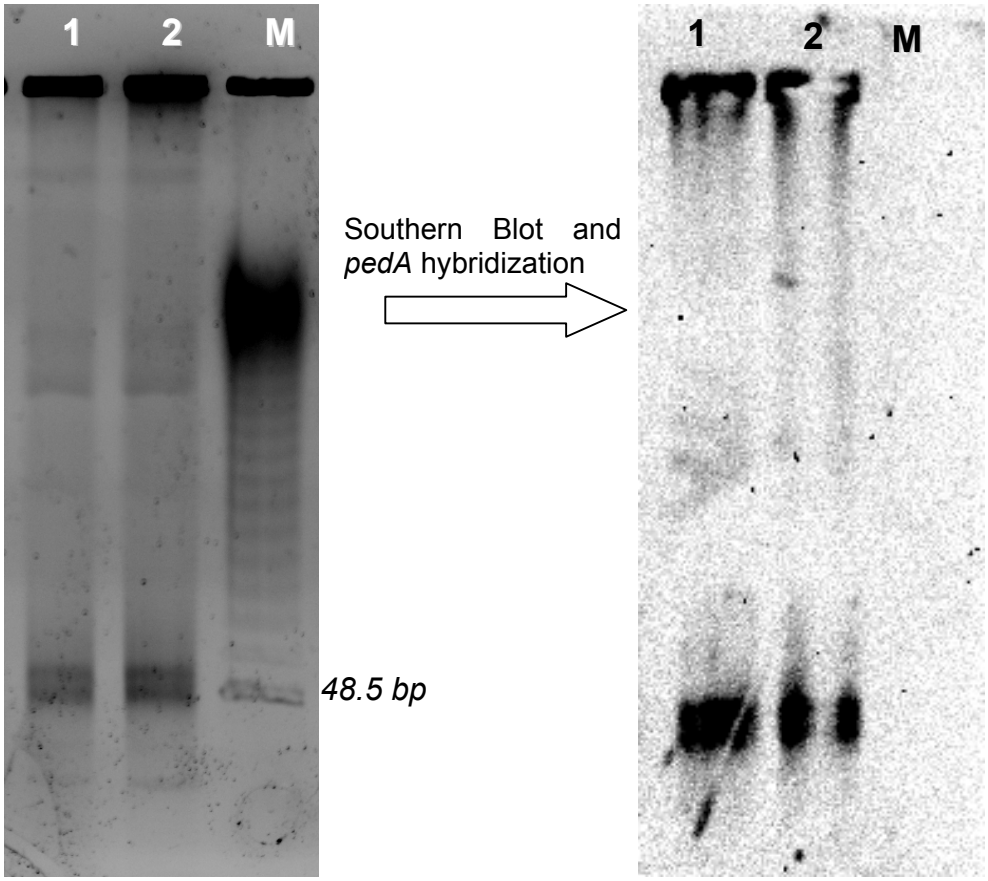


***Figure 26**: Pulsed-Field electrophoresis gel before DNA transfer via southern blotting (picture on the left) and Southern Blot membrane after hybdridization with pedA probe and CDP-Star detection (picture on the right).*
M: Lambda Ladder(Bio-Rad); 1-2: P.damnosus DNA plugs

As it is shown on Figure 26, hybridization with *pedA* probe gives three different signals on the membrane after chemiluminescence exposure. The enzyme alkaline phosphatase, fixed on the *pedA* probe, interacts with CDP-Star substrate to give light production visible on this figure. The first signal corresponds to the gel wells, band ζ of Figure 24. Light production of this band can be explained by the fact that a prominent part of *P.damnosus* DNA stayed in the gel wells. The two other signals correspond to bands α and β of the pulsed-field electrophoresis gel (see Figure 24). The *pedA* gene is thus present in one of these two bands which seems to be, as described above, the same plasmid in two different forms; α and β bands are respectively named plasmids α and β.

Finally, there is no light detection for the other DNA bands present on the membrane except weak signals created by DNA smear. By comparing the results given by this hybridization, it is now possible to confirm that *pedA* gene is present in either plasmid α or β.

3.6 Plasmid DNA extraction from pulsed-field electrophoresis gel

Following the results found after the hybridization with *pedA* probe, it has been possible to determine the location of DNA bands containing *pedA* gene on the pulsed-field electrophoresis gel. As it is shown on Figure 26, *pedA* gene is present on α and β plasmids. This is why an extraction of these two DNA bands has been realized in order to isolate these two plasmids. DNA bands α and β from Figure 24, corresponding to the two different plasmids containing *pedA* gene, have then been extracted from the agarose gel and purified using different kits. However these plasmids high size about 50 kbp make it a critical step. Indeed, plasmid extraction of such high size is difficult and give low DNA concentration. This is why optimization of the plasmids extraction was needed; several strategies have been developed as described below.

NucleoSpin® Extract II (Macherey-Nagel) was the first kit used to extract α and β plasmids from the agarose gel after pulsed-field electrophoresis; it shows a DNA concentration of about 5.2 µg/ml. Approximately 4.7 µg/ml DNA concentration has been found for a plasmid extraction with JetSorb kit (Promega). Using NucleoTrap® kit (Macherey-Nagel), DNA concentration is below 1.5 µg/ml. The last strategy use to extract these two plasmids was to, first digest plasmids into the agarose plugs and then, extract the fragments with NucleoSpin® Extract II kit (Macherey-Nagel) but it gave a low DNA concentration of about 2.5 µg/ml. These results are summarized on Table 5.

Table 5: DNA concentration after plasmids extraction and purification with different kits.

Essay	[ADN] µg/ml
plasmid α Extract II	5.25
plasmid β Extract II	5.20
plasmid α NucleoTrap	1.35
plasmid β NucleoTrap	1.35
plasmid α JetSorb	4.75
plasmid β JetSorb	4.70
plasmid α DNA plugs	2.70
plasmid β DNA plugs	2.35

Considering these results, the first strategy developed for this diploma work has been NucleoSpin® Extract II (Macherey-Nagel) since it has the higher DNA concentration.

3.7 Strategy 1: Plasmids α and β extracted from P.damnosus pulsed-field electrophoresis gel with NucleoSpin® Extract II kit (Macherey-Nagel).

3.7.1 pedA PCR-amplification for plasmids α and β

In order to control that *pedA* gene is really present in plasmids α and β extracted from the pulsed-field electrophoresis gel, Dot blotting and hybridization with *pedA* probe has been realized on these samples. The result of this experiment is shown on Figure 27.

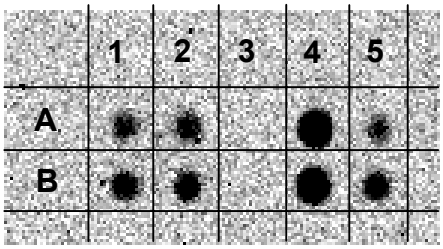


***Figure 27**: pedA hybridization for plasmids α and β, extracted from the PFGE gel, after Dot blotting.*
A1/B1 =plasmid α; A2/B2= plasmid β; ¸ A3/B3= K¯; A4/B4= pZErO-2; A4/B4= P.damnosus plasmid DNA extracted with Wizard Plus kit (Promega); A5/B5= P.damnosus genomic DNA extracted with NucleoSpin Tissue (Macherey-Nagel).

As shown on Figure 27, genomic DNA extracted from *P.damnosus* culture with NucleoSpin Tissue kit (Macherey-Nagel) and plasmid DNA extracted from *P.damnosus* culture with Wizard Plus SV Minipreps kit (Promega) indicate positive response to hybridization with *pedA* probe as well as for plasmids α and β; this result confirms the fact that *pedA* gene is part of these DNA. Concerning bands A3 and B3, they correspond to the negative control composed of PCR water, ethanol and NaOH; solution used to prepared the samples for Dot blotting. The result of this control is negative to *pedA* hybridization.

3.7.2 Cloning inserts into pZErO-2

Once the plasmids α and β have been extracted, quantified and controlled, cloning of digested plasmids can be realized into pZErO-2 vector in order to realize a plasmid bank after electroporation into *E.coli* cells and screening on LB-kanamycin plates.

pZErO-2 vector has a size of 3297 bp with a Multiple Cloning Site located between bases 269 and 381. The small insert, of unknown size, introduced into the MCS has the property to disrupt expression of *ccd*B gene. The endonucleases chosen for the parallel cloning strategy, *Eco*RI and *Hind*III, have a unique recognition site on this MCS situated at respectively 325 and 276 bp; a digestion with these enzymes used separately allows to linearize pZErO-2 vector into a unique fragment. These two enzymes also have the property to have no recognition sites in *pedA* gene; this gene will not be digested and can be detected by hybridization with *pedA* probe or by PCR screening with pedF and pedR primers after cloning and electroporation.

pZErO-2 vector has then been digested with *Eco*RI and *Hind*III to check if it gives a linearized unique fragment. A theoretical digestion on a 0.7% agarose gel has first been realized with REBsites in order to give the wanted results. Then, a practical DNA migration of pZErO-2 digested with respectively *Eco*RI and *Hind*III has been made by electrophoresis on a 0.7% agarose gel. The theoretical and practical results of these experiments are visible on Figure 28.

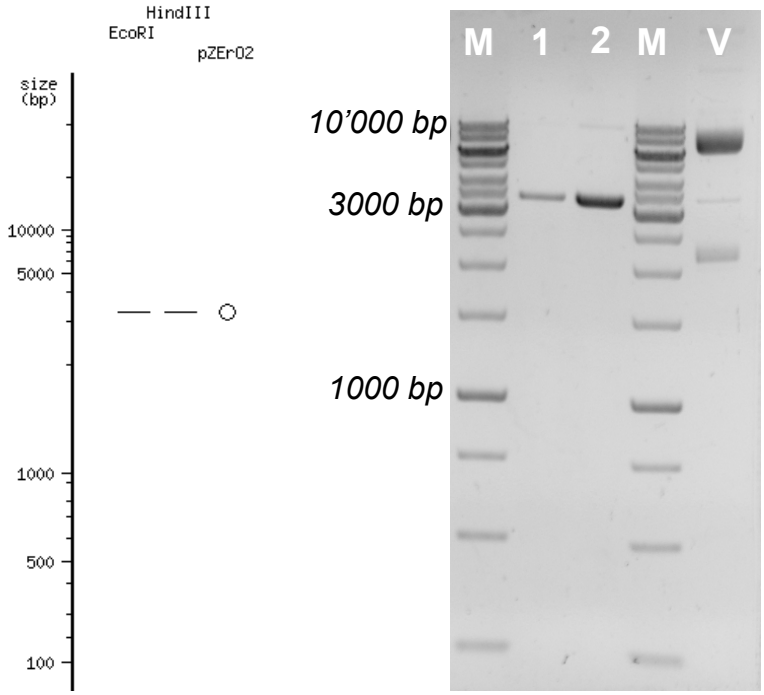


Figure 28: *Electrophoresis on 0.7% agarose gel of digested p-ZErO-2 vector with HindIII and EcoRI theoretical (picture on the left) and practical (picture on the right)*
M: GeneRuler 1 kb DNA ladder (Fermentas); 1: pZErO-2 digested with EcoRI; 2: pZErO-2 digested with HindIII; V: pZErO-2 vector.

Results given by the REBsites simulation, visible on the left side of Figure 28, show a unique linearized fragment situated around 3300 bp for pZErO-2 digestion using *Eco*RI and respectively *Hind*III. Simulation of pZErO-2 migration is also situated around 3300 bp and but is still circular, it should be higher on the gel.
Concerning electrophoresis of digested pZErO-2 vector, shown on the right side of Figure 28,on lanes 1 and 2, two thick bands are observable respectively with *Eco*RI and *Hind*III. The plasmid digested with *Eco*RI shows a thick band around 3400 bp and with *Hind*III around 3300 bp.
For pZErO-2 migration, shown on the right side of Figure 28, lane V, the DNA band is situated around 4000 bp but cannot be precisely determined since the plasmid is still in a circular form. As said before, its migration is slowed down because of its circular form. Considering this remark, this band corresponds to pZErO-2 circular vector. Two other weak bands are detectable at 3650 and 1900 bp and correspond respectively to pZErO-2 linearized and supercoiled.

The plasmids α and β fragments have then been ligated into this linearized pZErO-2 vector with T4 DNA ligase. In order to have enough quantity of insert and allow cloning of all the plasmid inserts into pZErO-2 vector, Equation 1 was used to calculate the exact amount of insert needed with an average insert size of 1 kbp.

$$\text{x ng insert} = \frac{(2)\,(\text{bp insert})\,(10\text{ ng linearized pZErO}^{\text{™}}\text{-2})}{(3297\text{ bp pZErO}^{\text{™}}\text{-2})} = \frac{(2)\cdot(1000bp)\cdot(10ng)}{(3297bp)} \approx 6.1ng$$

Equation 3: *Formula to calculate amount of insert needed for ligation with 10 ng of pZErO-2.*

A control of the ligation has been realized via an electrophoresis, as shown on Figure 29 to see if this step did work correctly.

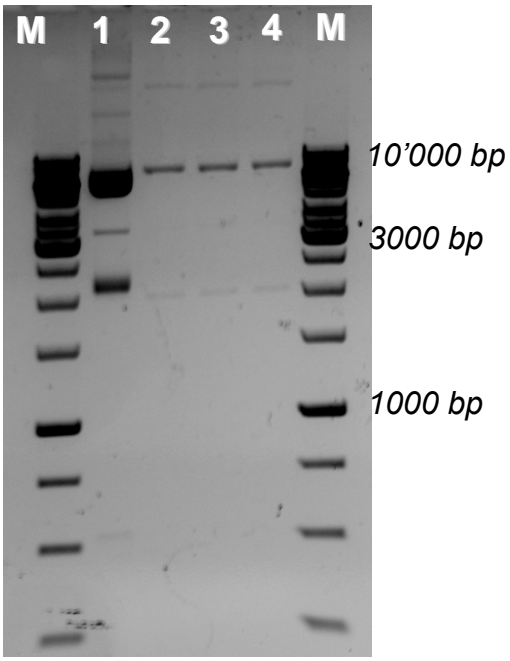


Figure 29: *Electrophoresis on 1% agarose gel of ligation results between plasmids α and β fragments and pZErO-2 vector.*
M: GeneRuler 1 kb DNA ladder (Fermentas); 1: P.damnosus DNA; 2: p-ZerO-2 self-ligation; 3: Ligation plasmid α fragments and pZErO-2; 4: Ligation plasmid β fragments and pZErO-2.

On Figure 29, ligation controls of recombinant plasmids are visible. Lane 1 corresponds to *P.damnosus* DNA. Lane 2 is the self-ligated pZErO-2 after restriction with *Eco*RI; it shows a thick band at about 8000 bp but still in a circular form, which migrates slower. No difference is visible between self-ligated pZErO-2 vector, lane 2, and recombinant plasmids, lanes 3 and 4. This control does not allow thus to confirm that ligation did work correctly. However, electroporation has been performed.

3.7.3 Insertion by electroporation of recombinant vectors into E.coli cells and screening

After ligation of plasmids α and β fragments into pZErO-2 vector, an insertion by electroporation was needed to insert these recombinant plasmids into *E.coli* TOP10 cells in order to construct the searched plasmid library.

During electroporation of recombinant plasmids into *E.coli* TOP10, impulsion time has been measured to verify the performance of the transformation. The ideal condition for DNA transfer and cells surviving is around 5 ms. Three different electroporations have been achieved for transformation of plasmid α fragments; impulsion time was between 4.80 and 5.10 ms. Three other electroporations have been achieved for transformation of plasmid β fragments; impulsion time was between 4.10 and 5.20 ms. These recombinant cells have then been plated onto LB-kanamycin plates and incubated in order to perform a screening on LB-kanamycin plates.

Insertion of pZErO-2 vector into *E.coli* TOP10 cells has been tested; time impulsion for this positive control was 4.90 ms. After plating the positive control onto LB-kanamycin plates and incubation, around 150 colonies were counted and reported in Table 6. In order to check if these *E.coli* TOP10 cells do not grow on this kanamycin medium, *E.coli* cells have been plated onto a LB-kanamycin plate and incubated. The result of these controls is shown in Table 6.

Table 6: Colonies counted after electroporation and screening using plasmids α and β strategy.

Essay	Insertion in *E.coli* cells	Colonies
1	pZErO-2 linearized + *P.damnosus* plasmid α	0
1 conc		0
2		8
2 conc		113
3		3
3 conc		0
4		0
4 conc		13
1	pZErO-2 linearized + *P.damnosus* plasmid β	0
1 conc		12
2		1
2 conc		11
3		6
3 conc		13
4		0
4 conc		20
K^+	pZErO-2 vector	150
K^+ conc		>300
K^-	-	0
K^- conc		0

By observing Table 6, it can be deduced that electropration succeeded. In fact, *E.coli* TOP10 cells growth onto LB-kanamycin plates does not show any colonies; kanamycin resistance is thus absent from these cells. However after electroporation, growth onto LB-kanamycin plates of *E.coli* TOP10 cells transformed with pZErO-2 vector shows around 150 colonies; kanamycin resistance, coming from pZErO-2 vector insertion, is this time present in the *E.coli* cells. However, the small quantity of colonies found on the positive control shows that electroporation is not yet optimal.

3.7.4 Creation of a plasmid library

As it has not been possible to prove that *pedA* gene was present in either plasmid α or β, two different plasmid libraries with respectively each plasmid had to be created. Once recombinant plasmids have been created and transformed into *E.coli* cells, a reliable plasmid bank that contains a random collection of the fragments from plasmids α and β was produced. To do so, Clarke and Carbon's formula (Equation 2) was used to estimate the clones number needed to get all the fragments of plasmids α and β into the plasmid library. Number of clones N can be thus calculated with a probability of 95% (P=0.95) and a ratio between the plasmid size of about 50 kbp and the cloned fragments of about 1 kbp (n=50).

$$N = \frac{\ln(1-P)}{\ln\left(1-\frac{1}{n}\right)} = \frac{\ln(1-0.95)}{\ln\left(1-\frac{1}{50}\right)} \cong 150 \text{ clones}$$

Equation 2: *Clark and Carbon's formula*

Therefore, 150 clones per plasmid would be needed in order to obtain a full plasmid bank. After purification of all the 150 clones and DNA extraction, it will be possible with PCR screening and/or hybridization with *pedA* probe to detect if recombinant plasmids contain the searched *pedA* gene.

E.coli TOP10 cells transformed with recombinant plasmids have then been plated onto LB-kanamycin plates and incubated. This screening allows to isolate cells containing the kanamycin resistance gene. Only cells transformed with either recombinant plasmids or self-ligated pZErO-2 vector can grow on this medium containing antibiotic.

3.7.5 DNA extraction from clones

After transformation and screening on LB-kanamycin plates, 160 clones have been isolated in 1.5 ml LB-kanamycin medium in order to extract their DNA and analyse it. Since the number of clones is really high, clones have been regrouped by 10, as shown on Table 7, to minimize the manipulations during PCR screening.

Table 7: Series of clones culture and restriction enzyme used, using Extract II kit strategy.

Series	Clones	Restriction enzyme used	Series	Clones	Restriction enzyme used
1	α1-α10	*Eco*RI	9	α 81- β90	*Hind*III
2	α11-α20	*Eco*RI	10	α 91- β100	*Hind*III
3	α21-α30	*Eco*RI	11	β1- β10	*Hind*III
4	α31-α40	*Eco*RI	12	β11- β20	*Hind*III
5	α41-α50	*Eco*RI	13	β21- β30	*Hind*III
6	α51-α60	*Eco*RI	14	β31- β40	*Hind*III
7	α61-α70	*Eco*RI	15	β41- β50	*Hind*III
8	α71-α80	*Eco*RI	16	β51- β60	*Hind*III

Genomic DNA has then been extracted from these 16 solutions, regrouping 160 clones, using NucleoSpin Tissue kit (Macherey-Nagel).

3.7.6 PCR screening

After DNA extraction of every purified clone, a PCR screening has been realized with pedF and pedR primers in order to find which plasmids contain an insert with the desired *ped*A gene. If a serie is positive after *pedA* PCR-amplification, the ten bacterial cultures of the serie have to be tested separately with a new PCR screening to find exactly which culture is positive to *pedA* amplification. Using this system, number of PCR screening has been divided by ten.
After the PCR screening step, electrophoresis on 1% agarose gels were realized in order to detect which streack possess *pedA* gene. One of this electrophoresis is shown on Figure 30 after detection under UV (GelDoc 100).

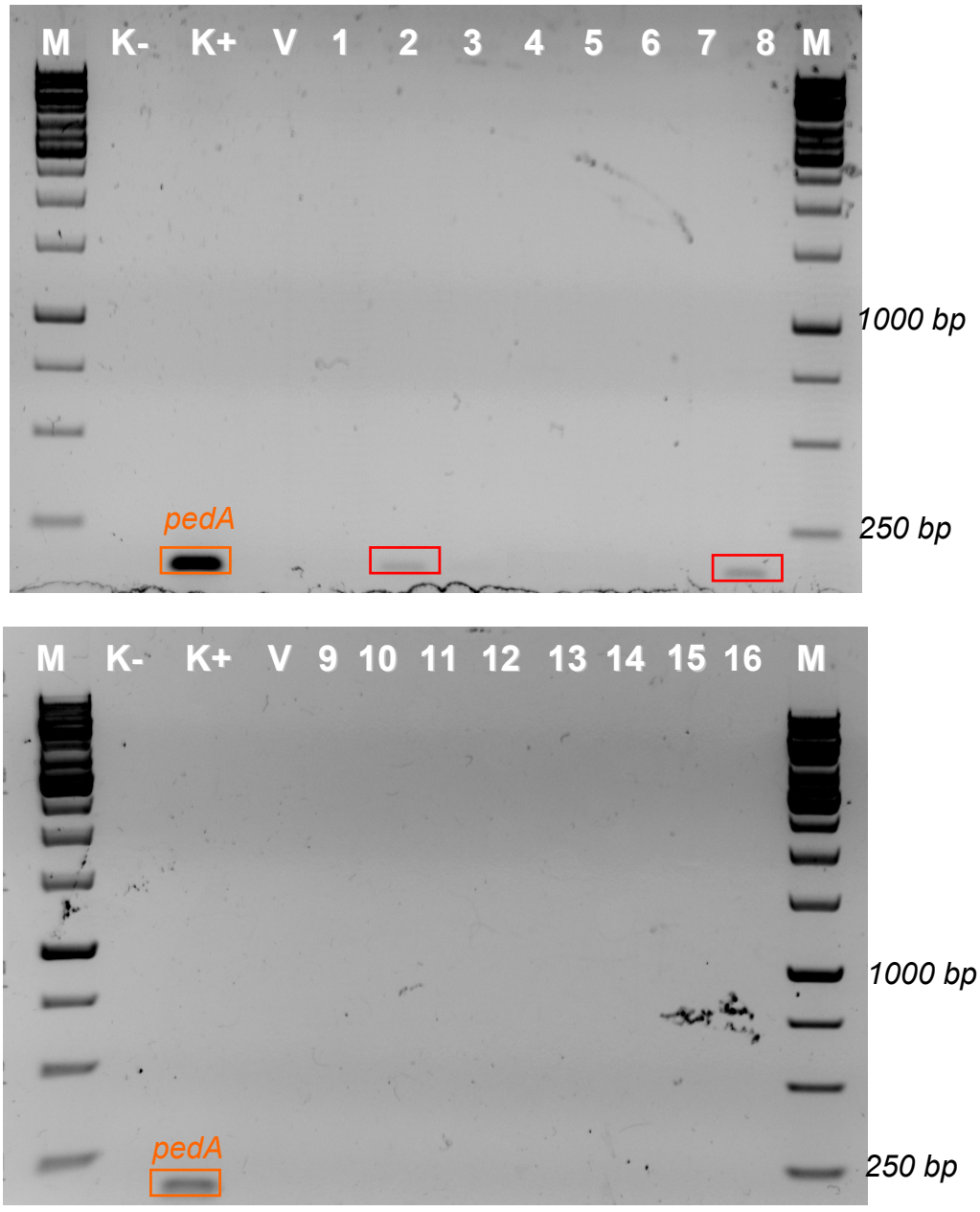


Figure 30: *PCR screening of the 16 series.*
M: GeneRuler 1 kb DNA ladder (Fermentas); K^-: PCR water (negative control); K^+: P.damnosus (positive control); V: Vector pZErO-2; 1-16: Series of ten plasmids.

As it is observable on Figure 30, the negative control, constituted of mastermix with PCR water, shows no *pedA*-amplification. In contrary, the positive control, constituted of *P.damnosus* DNA and mastermix, reveals an amplification of *pedA* gene. The final control of this PCR screening is, on lane V, vector pZErO-2 with mastermix; there is no *pedA* signal and therefore this plasmid does not contain *pedA* gene.
Concerning the clones series analyzed, they show two positive series to *pedA* amplification: series 2 and 8 as shown on Table 8. These two series are composed of plasmid α using *Eco*RI for the restriction strategy.

Table 8: Summary of *pedA* PCR-amplification results of series 1 to 16.

Series	*ped*A PCR-amplification	Series	*ped*A PCR-amplification
1	☒	9	☒
2	☑	10	☒
3	☒	11	☒
4	☒	12	☒
5	☒	13	☒
6	☒	14	☒
7	☒	15	☒
8	☑	16	☒

Since there have been several contamination problems with plasmids containing *pedA* gene during the PCR amplification, a confirmation that these two clone series have the *pedA* gene has been realized via Dot-blotting and hybridization with *pedA* probe.

3.7.7 Dot-blot and hybridization with pedA probe

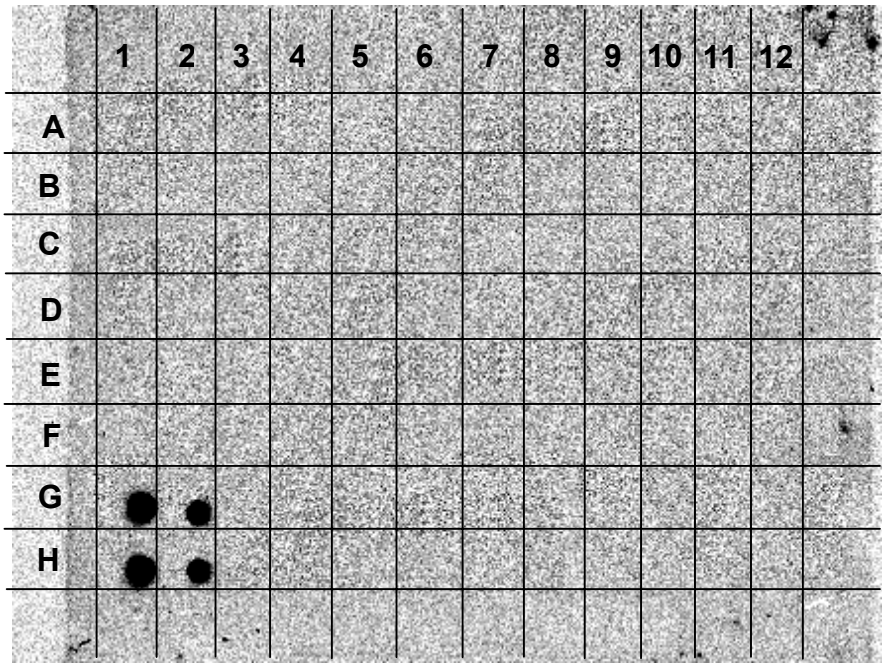


Figure 31: *Dot blot membrane after hybridization with pedA probe and CDP-star detection of the series 1 to 16.*

Samples: *A1/C1= Sample 1; A2/C2= Sample 2; A3/C3= Sample 3; A4/C4= Sample 4; A5/C5= Sample 5; A6/C6= Sample 6; A7/C7= Sample 7; A8/C8= Sample 8; A9/C9= Sample 9; A10/C10= Sample 10; D1/E1= Sample 10; D2/E2= Sample 11; D3/E3= Sample 12; D4/E4= Sample 13; D5/E5= Sample 14; D6/E6=Sample 15; D7/E7= Sample 16.*

Controls: *G1/H1= P.damnosus plasmid DNA; G2/H2= P.damnosus genomic DNA; G4/G4= K^-; G5/H5= pZErO-2.*

Confirmation via Dot Blot and pedA hybridization techniques shows no *pedA* response for all of the 16 clones series; there is therefore no recombinant plasmids containing pedA gene in these 160 clones. Results are summarized on Table 9.

Table 9: Summary of *pedA* hybridization results of 1 to 16.

Series	***pedA* hybridization**	Series	***pedA* hybridization**
K^-	☒	7	☒
pZErO-2	☒	8	☒
P.damnosus plasmid DNA	☑	9	☒
P.damnosus genomic DNA	☑	10	☒
1	☒	11	☒
2	☒	12	☒
3	☒	13	☒
4	☒	14	☒
5	☒	15	☒
6	☒	16	☒

Since no results have been found using this strategy (NucleoSpin Extract II kit), a second strategy has been developed in order to create a plasmid library containing at least one *pedA* positive recombinant plasmid. This new strategy consists of a direct extraction of plasmid DNA from *P.damnosus* culture with Wizard Plus SV kit (Promega) in order to avoid extracting α and β plasmids from agarose gel after pulsed-field electrophoresis since DNA concentration is really low with all four kits used (see *3.7 Plasmids extraction from agarose gel*).

3.8 Strategy 2: Plasmid DNA extracted from P.damnosus culture with Wizard Plus SV (Promega) using EcoRI for its restriction strategy.

As strategy 1 using plasmids α and β extracted from the pulsed-field electrophoresis gel did not gave adequate results, a new strategy with *P.damnosus* total plasmid DNA has been used.

Table 10: DNA concentration for *P.damnosus* plasmid extracted with Wizard kit (Promega)

Essay	[ADN] µg/ml
Plasmid DNA Wizard (Promega)	70

P.damnosus plasmid DNA extraction has been realized using Wizard® Plus SV kit (Promega) from 1.5 ml *P.damnosus* culture incubated overnight. A restriction of *P.damnosus* plasmid DNA using *Eco*RI enzyme has then been perfomed to see which plasmids are digested by this enzyme. Results are shown on Figure 32.

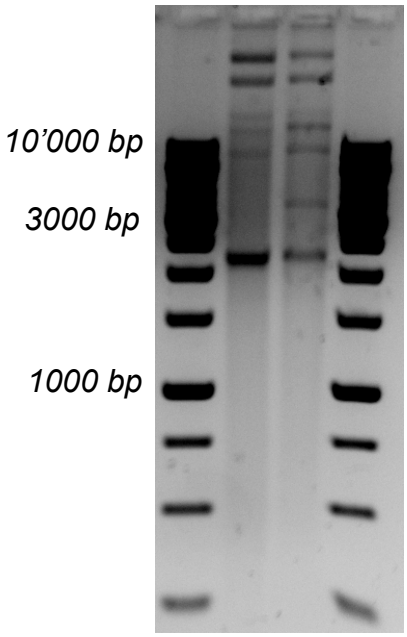


Figure 32: *P.damnosus plasmid DNA electrophoresis gel*
*M: GeneRuler 1 kb DNA ladder (Fermentas), 1: P.damnosus plasmid DNA, 2: P.damnosus plasmid DNA digested with Eco*RI.

On Figure 32, *P.damnosus* plasmid DNA, situated on lane 1, shows three different intensive bands. Two of them are superior to 10 kbp, one of this band could correspond to plasmid α and β; their size was estimated around 50 kbp using pulsed-field electrophoresis. The third band at 2200 bp could be another smaller *P.damnosus* plasmid. Other lighter bands situated around 10 kbp could be plasmids in different forms. On lane 2, plasmid DNA restriction with *Eco*RI is shown. Partial digestion of the three different plasmids is detectable since the bands corresponding to each plasmid are less intensive. However, according to these results, it is not possible to claim to *Eco*RI cuts plasmids α and β.
According to these results, the total size of *P.damnosus* plasmid DNA can be estimated around 100 kbp.

3.8.1 Cloning inserts into pZErO-2

P.damnosus plasmid DNA and pZErO-2 vector have been digested with *EcoR*I. After ligation of plasmid DNA fragments into pZErO-2 vector, electroporation was needed to insert these recombinant plasmids into *E.coli* TOP10 cells in order to construct the plasmid library.

During insertion of recombinant plasmids into *E.coli* TOP10, impulsion time has been measured to verify the performance of the electroporation. The ideal condition for DNA transfer and cells surviving is around 5 ms.
Four different electroporations have been achieved to insert recombinant plasmids into *E.coli* TOP10 cells; impulsion time was included between 4.20 and 4.80 ms. These recombinant cells have then been plated onto LB-kanamycin plates and incubated. Insertion of pZErO-2 vector into *E.coli* TOP10 cells has also been tested in order to detect if electroporation has worked; time impulsion for this positive control was 6.00 ms.

After plating the positive control onto LB-kanamycin plates and incubation, around 200 colonies were counted and reported in Table 11. In order to check if these *E.coli* TOP10 cells do not grow on kanamycin medium, *E.coli* cells have been plated onto LB-kanamycin plate and incubated. The result of these controls is shown in Table 11.

Table 11: Colonies after electroporation and screening for essays using *Eco*RI.

Essay	Insertion in *E.coli* cells	Colonies
1	pZErO-2 linearized + *P.damnosus* plasmid DNA fragments	13
1 conc		24
2		0
2 conc		174
3		10
3 conc		74
4		0
4 conc		7
K^+	pZErO-2 vector	200
K^+ conc		> 300
K^-	-	0
K^- conc		0

3.8.2 Creation of a plasmid library

Once recombinant vectors have been created and inserted into *E.coli* cells, a reliable plasmid library that contains a random collection of *P.damnosus* plasmid DNA fragments was produced. Clarke and Carbon's formula (Equation 2) was used to estimate the clones number needed to get all *P.damnosus* plasmid DNA fragments into the plasmid library. Number of clones N can be thus calculated with a probability of 95% (P=0.95) and a ratio between estimated plasmid DNA size of about 100 kbp and the cloned fragments of about 1 kbp (n=100).

$$N = \frac{\ln(1-P)}{\ln\left(1-\frac{1}{n}\right)} = \frac{\ln(1-0.95)}{\ln\left(1-\frac{1}{100}\right)} \cong 300 \text{ clones}$$

Equation 2: *Clark and Carbon's formula*

Therefore, at least 300 clones are needed in order to obtain a full plasmid bank, if EcoRI digests all *P.damnosus* plasmids. After purification of each clone and DNA extraction, it will be possible with PCR screening and/or hybridization with *pedA* probe to detect if recombinant plasmid contains the desired *pedA* gene.

3.8.3 DNA extraction from clones

After the electroporation and screening on LB-kanamycin plates, 82 clones have been isolated in 1.5 ml LB-kanamycin medium in order to extract their DNA and analyse it. Since the number of clones is high, clones have been regrouped by series of 8 in 1.5 ml tubes to minimize the manipulations, as shown on . DNA extraction, using NucleoSpin® Tissue kit (Macherey-Nagel), as been realized for these 10 samples.

Table 12: Series of clone cultures to perform DNA extraction before Dot blotting

Series	Clones	Series	Clones	Series	Clones	Series	Clones	Series	Clones
1	1	3	18	5	35	7	53	9	76
	2		19		37		54		78
	3		20		38		55		81
	4		21		39		56		82
	5		22		40		58		83
	6		23		41		60		84
	8		24		42		63		85
	9		25		43		64		
2	10	4	26	6	44	8	66	10	86
	11		27		45		69		87
	12		28		46		70		88
	13		29		47		71		89
	14		30		48		72		
	15		32		50		73		
	16		33		51		74		
	17		34		52				

These 82 clones have been conserved in glycerol 87% and stocked at –70°C for further analysis; see appendices *8.9 Plasmid library using EcoRI* for details.

3.8.4 Dot blot and hybridization with pedA probe

Once DNA has been extracted from the 10 different seires, regrouping 84 clones, Dot blotting and *pedA* hybridization has been performed. After CDP-Star detection and chemiluminescence expose for 20 min, the result is shown on Figure 33.

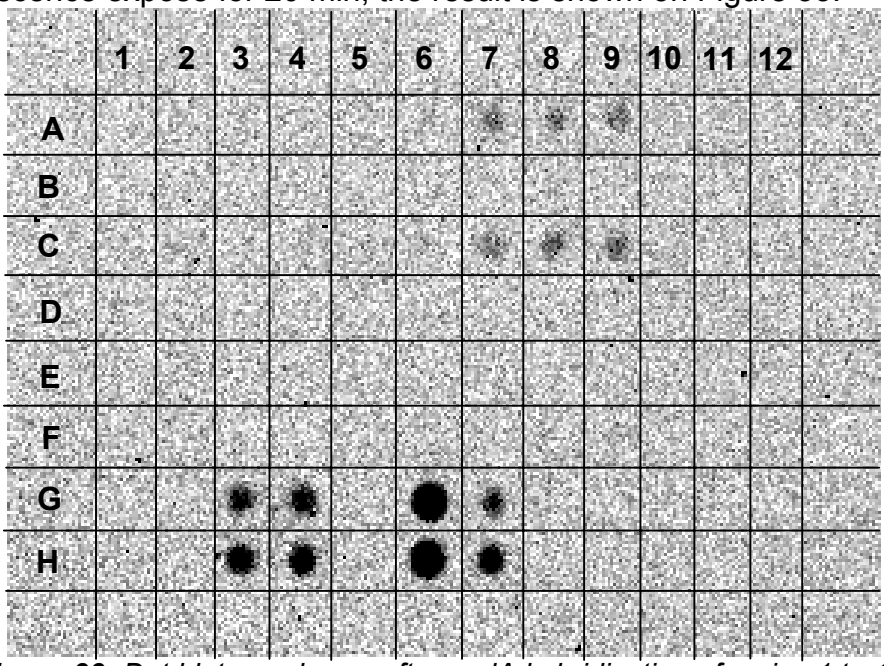


Figure 33: *Dot blot membrane after pedA hybridization of series 1 to 10.*

Samples: *A1/C1= Sample 1; A2/C2= Sample 2; A3/C3= Sample 3; A4/C4= Sample 4; A5/C5= Sample 5; A6/C6= Sample 6; A7/C7= Sample 7; A8/C8= Sample 8; A9/C9= Sample 9; A10/C10= Sample 10.*

Controls: *G1/H1= K-; G2/H2= pZErO-2; G3/H3=plasmid α; G4/H4= plasmid β; G6/H6= P.damnosus plasmid DNA; G7/H7= P.damnosus genomic DNA.*

As shown on Figure 33, *P.damnosus* genomic DNA extracted with Wizard Plus SV Minipreps kit (Promega), *P.damnosus* plasmid DNA extracted with NucleoSpin Tissue (Macherey-Nagel) indicate positive response to hybridization with *pedA* probe; this confirms that *pedA* gene is present in these DNAs. Plasmids α and β extracted form pulsed-field electrophoresis gel have also been tested to confirm the presence of *pedA* gene. Concerning the samples, three positive responses out of ten have been found: samples 7, 8 and 9. These results are illustrated on Table 13.

Table 13: Summary of results for dot blot membrane *pedA* hybridization of series 1 to 10.

Samples	*pedA* hybridization	Samples	*pedA* hybridization
K⁻	☒	Sample 3	☒
pZErO-2	☒	Sample 4	☒
P.damnosus plasmid DNA	☑	Sample 5	☒
P.damnosus genomic DNA	☑	Sample 6	☒
Plasmid α	☑	Sample 7	☑
Plasmid β	☑	Sample 8	☑
Sample 1	☒	Sample 9	☑
Sample 2	☒	Sample 10	☒

As these three samples are positives to *pedA* hybridization, a DNA extraction had to be performed for every clone among series 7, 8 and 9; this means clones 53 to 85 (see Table 13). Once again, Dot blotting and *pedA* hybridization has been performed to determine exactly which clones possess the *pedA* gene. After CDP-Star detection and exposure in chemiluminesence for 20 min, the picture is shown on Figure 34.

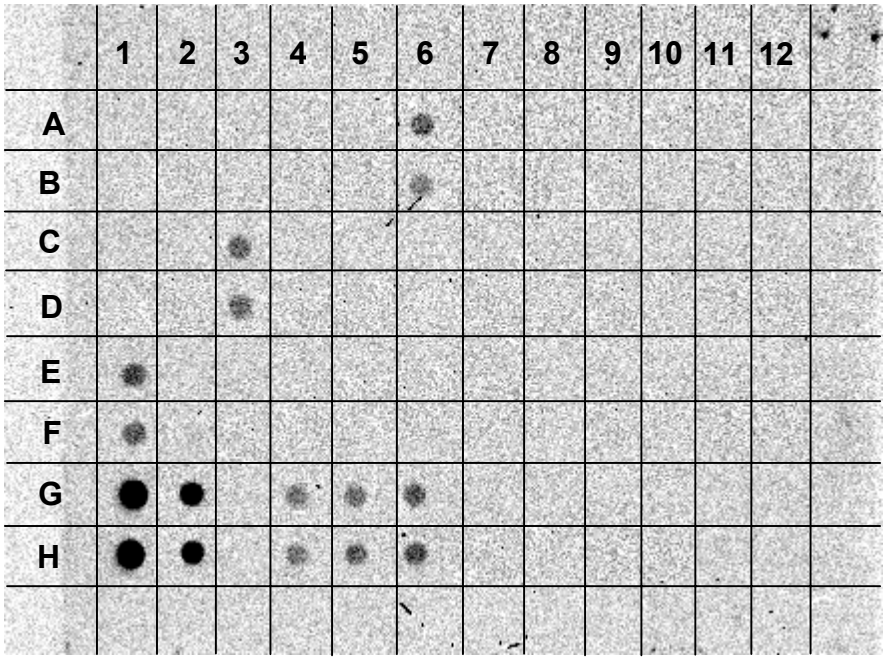


Figure 34: *Dot blot membrane after hybridization with pedA probe and CDP-star detection of the clones from series 7, 8 and 9.*

Sample 7: A1/B1= clone 53; A2/B2= clone 54; A3/B3= clone 55; A4/B4= clone 56; A5/B5= clone 58; A6/B6= clone 60; A7/B7= clone 63; A8/B9= clone 64.

Sample 8: C1/D1= clone 65; C2/D2= clone 66; C3/D3= clone 70; C4/D4= clone 71; C5/D5= clone 72; C6/D6= clone 73; C7/D7= clone 74.

Sample 9: E1/F1= clone 76; E2/F2= clone 78; E3/F3= clone 81; E4/F4= clone 82; E5/F5= clone 83; E6/F6= clone 84; E7/F7= clone 85.

Controls: G1/H1= P.damnosus plasmid DNA; G2/H2= P.damnosus genomic DNA; G3/H3= K-; G4/H4= Sample 7; G5/H5= Sample 8; G6/H6= Sample 9.

As shown on Figure 34, *P.damnosus* genomic DNA extracted with Wizard Plus SV Minipreps kit (Promega) and *P.damnosus* plasmid DNA extracted with NucleoSpin Tissue (Macherey-Nagel) indicate again a positive respond to hybridization with *pedA* probe as well as series 7, 8 and 9; this result confirms the fact that *pedA* gene is part of these three series of DNA.

For serie 7, only clone 60 shows *pedA* hybridization. The seven others clones of the serie are negative. Concerning serie 8, one among the seven clones is positive to *pedA* hybridization: clone 70. Finally, for serie 9, clone 76 is positive and the six other clones are negative. These results are summarized on Table 14.

Table 14: Summary of results for dot blot membrane *pedA* hybridization of clones from series 7, 8 and 9.

Samples	*pedA* hybridization	Samples	*pedA* hybridization	Samples	*pedA* hybridization
K^-	☒	K^-	☒	K^-	☒
pZErO-2	☒	pZErO-2	☒	pZErO-2	☒
P.damnosus plasmid DNA	☑	*P.damnosus* plasmid DNA	☑	*P.damnosus* genomic DNA	☑
P.damnosus genomic DNA	☑	*P.damnosus* genomic DNA	☑	*P.damnosus* genomic DNA	☑
Clone 53	☒	Clone 65	☒	Clone 76	☑
Clone 54	☒	Clone 66	☒	Clone 78	☒
Clone 55	☒	Clone 70	☑	Clone 81	☒
Clone 56	☒	Clone 71	☒	Clone 82	☒
Clone 58	☒	Clone 72	☒	Clone 83	☒
Clone 60	☑	Clone 73	☒	Clone 84	☒
Clone 63	☒	Clone 74	☒	Clone 85	☒
Clone 64	☒				

3.9 Creation of a genetic map for pedA positive plasmids

As three different clones out of the 82 clones analyzed show *pedA* hybridization, restriction map have to be created to built the genetic map for each of the three *pedA* positive clones. To do so, plasmid DNA has been extracted from LB-kanamycin *P.damnosus* with Wizard Plus SV kit (Promega). Several restrictions have then been realized on each three recombinant plasmids, as well as on pZErO-2 vector, in order to built a restriction map of each plasmid. The restriction strategy has been chosen following the number of recognition sites on pZErO-2 vector and *pedA* gene as shown on Table 15.

Table 15: Restriction enzymes, and their recognition sites, chosen for building a genetic map of the three *pedA* positive recombinant plasmids.

Restriction enzymes	Recognition sites	Cuts in pZErO-2	Cuts in *pedA*
*Ase*I	5′...AT▼TAAT...3′ 3′...TAAT▲TA...5′	2	1
*Eco*RI	5′...G▼AATTC...3′ 3′...CTTAA▲G...5′	1	0
*Rsa*I	5′...GT▼AC...3′ 3′...CA▲TG...5′	4	2
*Sap*I	5′...GCTCTTC(N)$_1$▼...3′ 3′...CGAGAAG(N)$_4$▲...5′	3	0
*Sph*I	5′...GCATG▼C...3′ 3′...C▲GTACG...5′	2	0

Using REBsites, a theoritical restriction map of pZErO-2 vector has been created and illustrated on Figure 35.

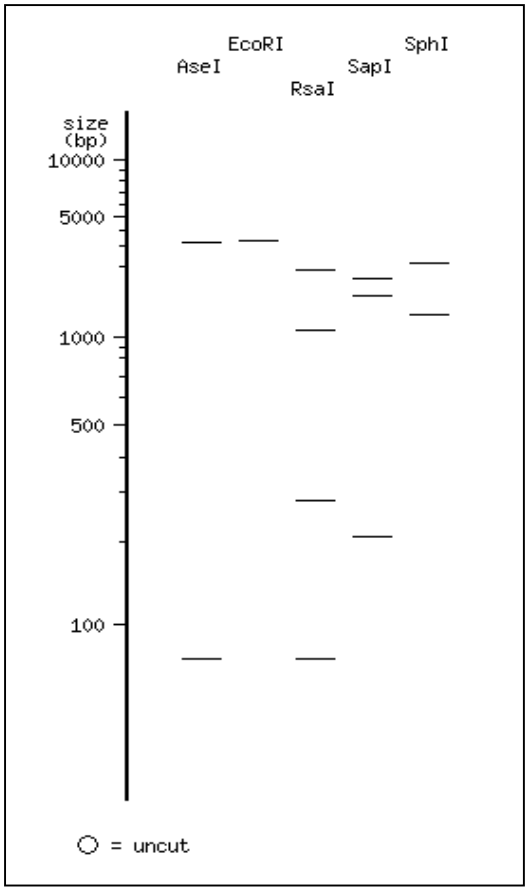


Figure 35: *Theoretical pZErO-2 restriction with chosen enzymes on 1% agarose gel.*

As shown on Figure 35, two pZErO-2 fragments of 3238 bp and 59 bp are obtained with *Ase*I restriction. With *Eco*RI, a unique fragment of 3297 bp is visible. *Rsa*I cuts four times pZErO-2 vector to give 4 fragments of 1900 bp, 1058 bp, 279 bp and 60 bp. *Sap*I restriction gives three fragments of 1666 bp, 1421 bp and 210 bp. Finally, *Sph*I gives 2 fragments of 2078 bp an 1219 bp. Concerning *pedA* gene, only two enzymes out of the five chosen cuts the sequence. *Ase*I cuts a unique time and *Rsa*I twice the pedA gene, Using pDRAWN32® software, genetic maps of pZErO-2 and of *pedA* gene with the five chosen enzymes have been built as shown on as shown on Figure 36 and Figure 37.

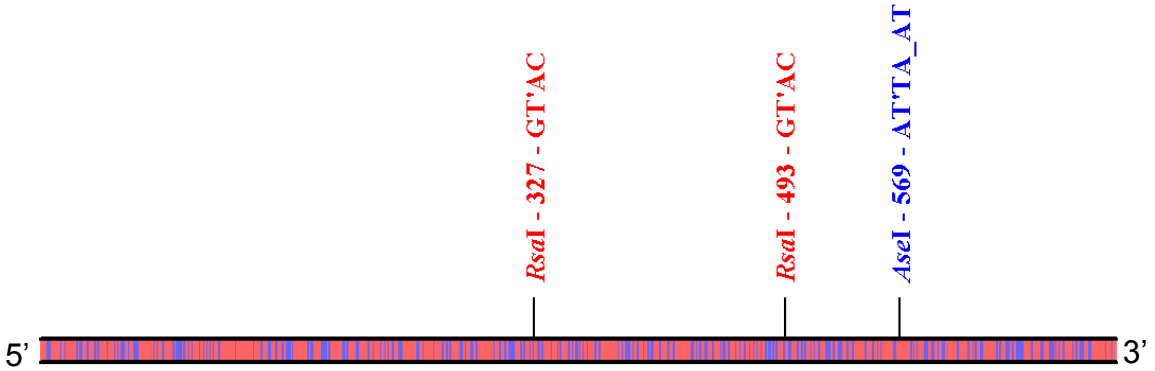


Figure 36: *pedA gene with recognition sites for the 5 enzymes chosen (pDRAWN32).*

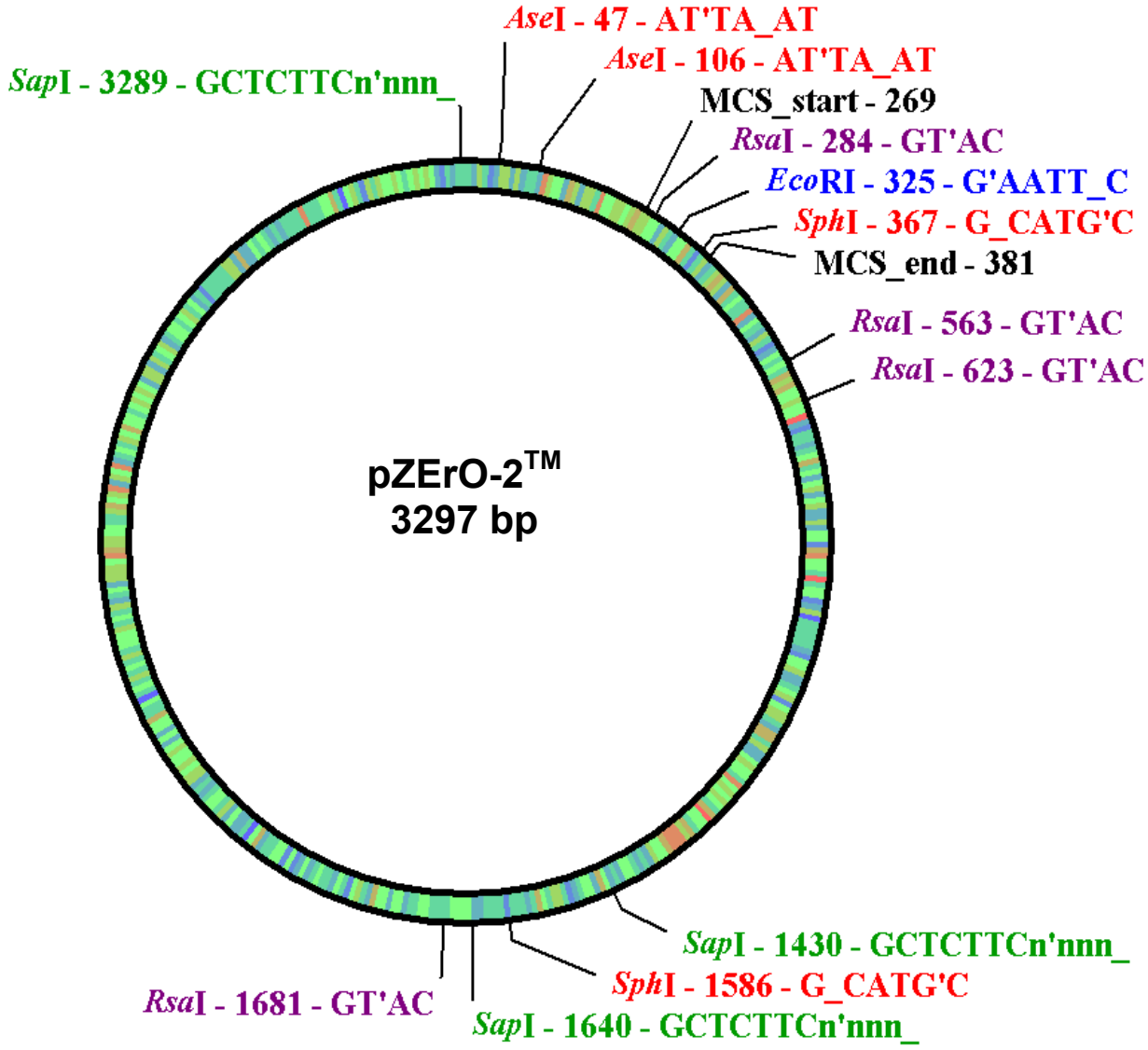


Figure 37: *pZErO-2 vector with recognition sites for the five chosen enzymes (pDRAWN32).*

As shown on Figure 37, pZErO-2 contains three recognition sites, out of the five enzymes chosen, in its Multiple Cloning Site. *Eco*RI recognition site is used to place in pZErO-2 vector the different fragments of *P.damnosus* plasmid DNA. As seen above, only three clones possess an insert with *pedA* gene: plasmids p60, p70 and p76. A genetic map is thus constructed for each of these three clones.

3.9.1 Genetic map creation of recombinant plasmid p60

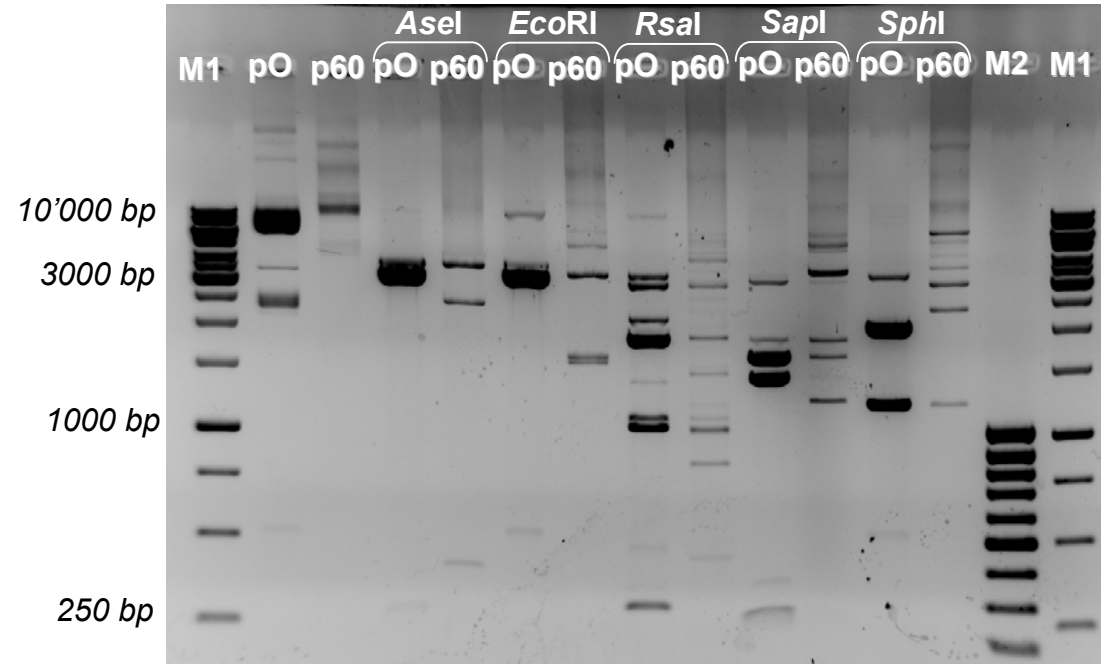


Figure 38: *Restriction of pZErO-2 vector and of recombinant plasmid p60 with the five chosen enzymes.*
M1: GeneRuler 1 kb DNA ladder (Fermentas), pO: pZErO-2 vector, p60: recombinant plasmid extracted from clone c60; M2: Ladder 100 bp (GeneRuler).

pZErO-2 and recombinant plasmid p60, extracted from clone 60, have been digested with the five chosen enzymes and regrouped on Figure 38. Lanes p0 and p60 correspond to pZErO-2 vector and plasmid p60 without digestion; three distinct bands are visible for pZErO-2 at 8000 bp, 3300 bp and 2400 bp corresponding to circular, linearized and supercoiled forms of the vector. Recombinant plasmid p60 possesses a thick band superior to 10 kbp, which seems to correspond to its circular form. According to these results, p60 is not from the same size than pZErO-2 and therefore, possesses an insert.

Concerning pZErO-2 restriction, *Ase*I should give two fragments of 3238 bp and 59 bp. Only one thick band is visible at 3200 bp and the second one is out of the gel. *Eco*RI should give one fragment at 3297 bp, even two if we consider the insert surrounded by two *Eco*RI recognition sites, but as it is so small, it is not considered in these results. One thick band at 3100 bp, corresponding to pZErO-2 linearized with *Eco*RI, and another one at 8000 bp corresponding to pZErO-2 vector not digested are visible for this digestion. *Rsa*I digestion gives too much fragments; it has been decided not to consider these results for building the genetic map of p60 as results are not logical. However, three of the four theoretical fragments that should be found with this enzyme are visible: 100 bp, 1000 bp and 300 bp. The fourth one, about 60 bp, is out of the gel. *Sap*I digestion gives four bands; 8000 bp corresponding to pZErO-2 circular and 1600 bp, 1350 bp and 200 bp corresponding to the three fragments that should be found with *Sap*I. *Sph*I digestion gives three distinct bands; one at 8000 bp corresponding again to pZErO-2 circular and two bands, 2000 bp and 1200 bp corresponding to the two fragments that should be found with *Sph*I.

These restriction pZErO-2 results are summarized on Table 16 with, in parallel, theoretical fragment sizes in order to compare them.

Table 16: Theoretical and practical DNA fragment sizes, in bp, after pZErO-2 restriction with the five chosen enzymes for p60 analysis.

	***Ase*I**		***Eco*RI**		***Rsa*I**	
	Theoretical	Practical	Theoretical	Practical	Theoretical	Practical
	3238	3200	3297	3000	1900	1800
	59	-			1058	1000
					279	300
					60	-
Total	*3297*	*3200*	*3297*	*3000*	*3297*	*3100*

	***Sap*I**		***Sph*I**	
	Theoretical	Practical	Theoretical	Practical
	1666	1600	2078	2000
	1421	1350	1219	1200
	210	200		
Total	*3297*	*3150*	*3297*	*3200*

Recombinant plasmid p60 digestion is also visible on Figure 38 with the five chosen enzymes. According to *pedA* hybridization, which assume that p60 contains pedA gene, an analysis between pZErO-2 and p60 restriction has been made.

*Ase*I restriction gives two thick bands, one at 3700 bp and another one at 2600 bp. The total size of these two fragments is 6300 bp; p60 size is therefore around 6300 bp. As pZErO-2 size is about 3300 bp, the insert size can be estimated at 3000 bp.
For p60 restriction, a recognition site for this enzyme is present on *pedA* gene and two others on pZErO-2 vector (see Figure 36 and Figure 37), but these two sites are so close to each other that it gives a fragment of 59 bp that is not visible on this gel. This is why only two fragments are detectable on the gel. By assuming that *pedA* gene is present on the insert, it can be localized by analyzing in details these results. The lengths between the two *Ase*I recognition sites on pZErO-2 and the *Eco*RI site, in which the insertion has been made, give 219 bp between *Ase*I site situated at 106 bp and *Eco*RI site situated at 325 bp and another 3019 bp between *Ase*I site situated at 47 bp and the same *Eco*RI site (see Figure 37). Therefore, it is not possible to have p60 fragment of 2400 bp situated between *Ase*I site 47 bp and *Eco*RI site 325 bp since it is smaller than 3019 bp. *Ase*I recognition site is then presume to be situated around 2180 bp on the insert. Since *Ase*I recognition site is part of *pedA* gene, it is assumed that *pedA* gene is localized on the insert between 1600 bp and 2300 bp as illustrated on Figure 39.

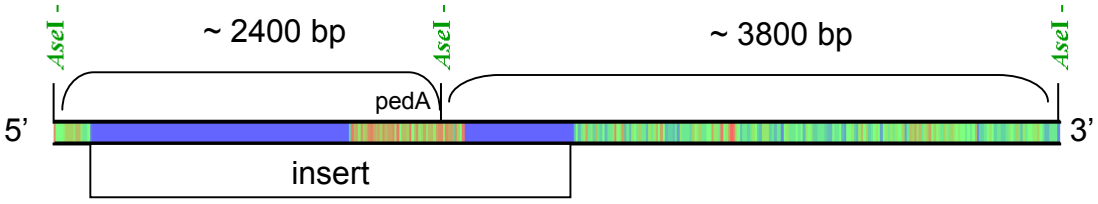


Figure 39: *Assumed pedA gene localization on p60 insert*

According to Figure 39, the two unknown regions of the insert (in blue), with *pedA* between them, should be of size 1600 bp and 1000 bp.

*Eco*RI restriction gives three different fragments of size 3100 bp, 1600 bp and 1500 bp, the total size of these three fragments is 6300 bp. By knowing that pZErO-2 size is 3298 bp, p60 insert can be assumed to be around 3000 bp.
As the insert as been inserted in pZERO-2 vector on *Eco*RI recognition site, p60 should possess two recognition sites, which surround the insert. Concerning *pedA* gene, it do not possess an *Eco*RI site (see Figure 36). As three fragments are found for *Eco*RI restriction, the third recognition site should be present on the unknown part of the insert. In addition, since p60 fragment of 3100 bp seems to correspond to pZErO-2 fragment of 3000 bp, the two others fragments, of 1600 bp and 1500 bp, may correspond to the insert cut in two.

*Sap*I restriction gives three fragments of size 3500 bp, 1600 bp and 1200 bp, a fourth one should be found at 200 bp but is not detectable on this gel. The total size of these fragments is 6300 bp which gives an insert of about 3000 bp since pZErO-2 is 3298 bp
pZErO-2 possesses three *Sap*I recognition sites (see Figure 37) and none for *pedA* gene (see Figure 36); the fourth site found on p60 can therefore be only present on the unknown part of the insert. p60 fragment of 1600 bp seems to correspond to pZErO-2 fragment of 1666 bp and p60 fragment 200 bp is not visible on the gel. pZErO-2 fragment of 1421 bp can be represented by two p60 fragments of 3500 bp and 1200 bp; the insert size should be about 3200 bp. However, exact localization of *Sap*I recognition site on the insert is not possible.

Finally, *Sph*I restriction gives three distinct fragments at 3000 bp, 2350 bp and 1200 bp for a total size of 6600 bp. Insert size should be around 3300 bp since pZErO-2 is 3298 bp. A fourth band is visible at 8000 bp and could correspond to circular p60.
As pZErO-2 vector has two *Sph*I recognition sites (see Figure 37), there is apparently a *Sph*I site on the unknown part of the insert since three fragments have been found. The third fragment of 1200 bp corresponds to same-sized pZErO-2 fragment and do not possess p60 insert. Concerning the two other fragments, of 3000 bp and 2350 bp, it seems that they both possess part of the insert at their extremities since pZErO-2 fragment corresponding to these two p60 fragments is 2000 bp. However, exact localization of *Sph*I recognition site on the insert is not possible.

According to these p60 restriction results, which are summarized on Table 17, it is possible to pretend that recombinant plasmid p60 possesses an insert of about 3 kbp and has an *Eco*RI, *Sap*I and *Sph*I recognition sites on its unknown part.

Table 17: DNA fragment sizes, in bp, after p60 restriction with *Ase*I, *Eco*RI, *Sap*I and *Sph*I.

***Ase*I**	***Eco*RI**	***Sap*I**	***Sph*I**
3800	3100	3500	3000
2400	1600	1600	2350
	1500	1200	1200
6300	*6200*	*6300*	*6600*

Next to the comparison of the pZErO-2 and p60 restriction results, it has been assume that the genetic map of p60 is as illustrated on Figure 40.

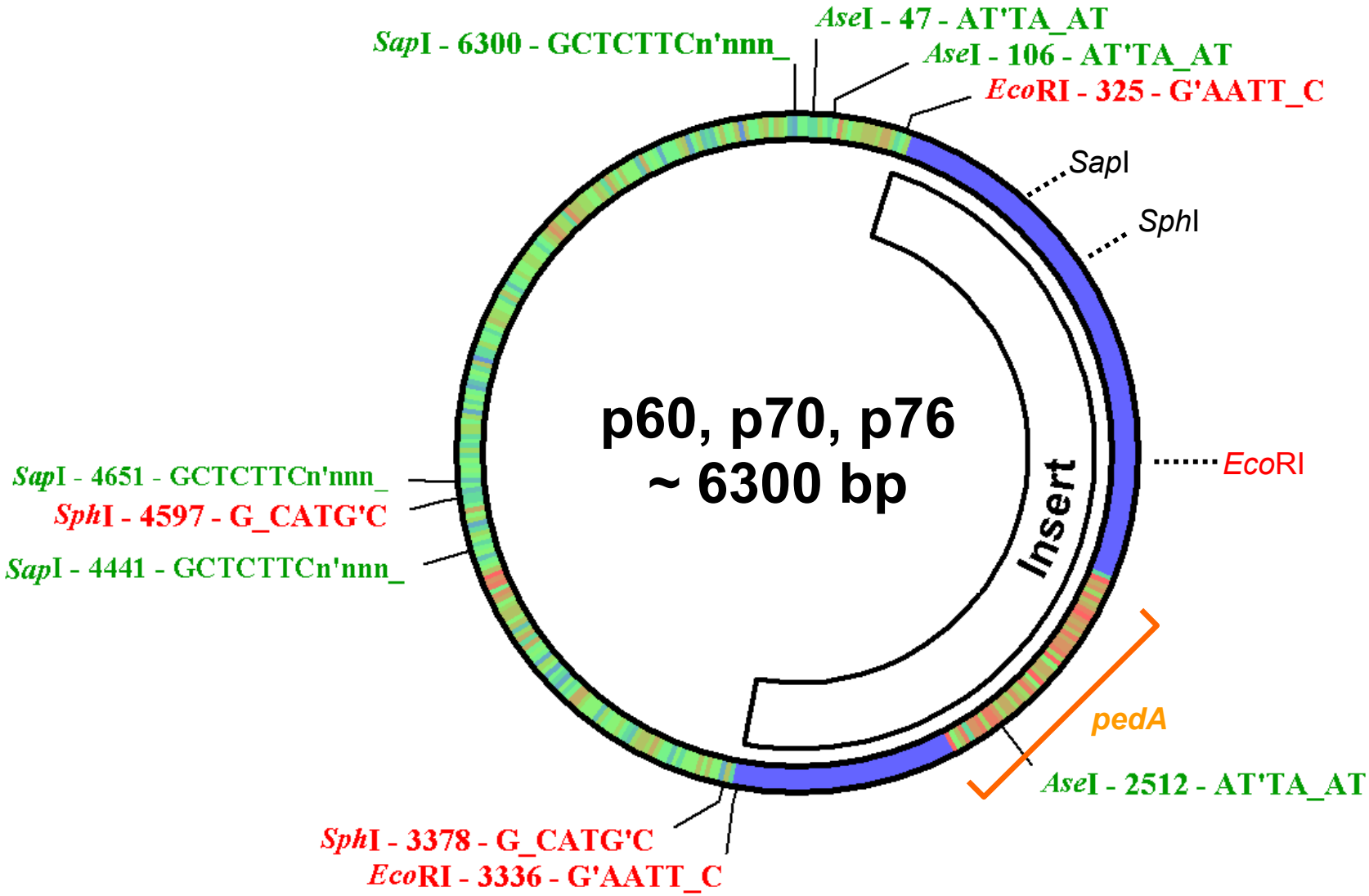


Figure 40: *Assumed p60 genetic map*

3.9.2 Genetic map creation of recombinant plasmid p70

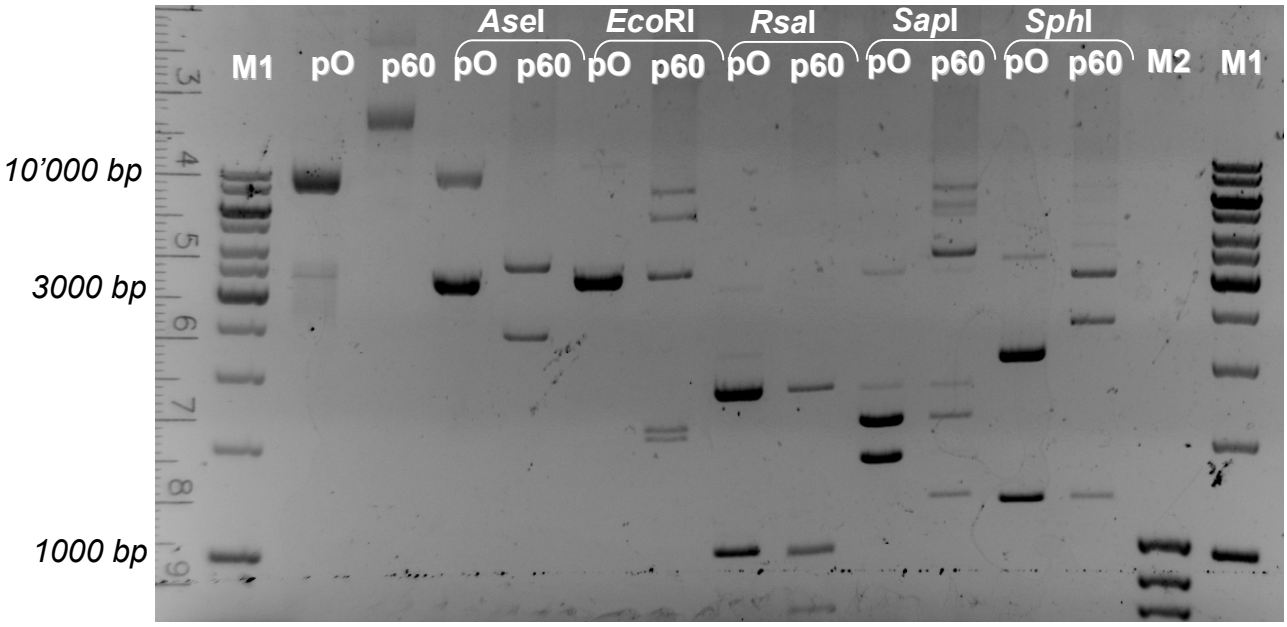


Figure 41: *Restriction of pZErO-2 vector and of recombinant plasmid p70 with the five chosen enzymes.*
M1: GeneRuler 1 kb DNA ladder (Fermentas), pO: pZErO-2 vector, p60: recombinant plasmid extracted from clone c60; M2: Ladder 100 bp (GeneRuler).

pZErO-2 and recombinant plasmid p70, extracted from clone 70, have been digested with the five chosen enzymes and regrouped in Figure 41. Analysis of pZErO-2 restriction gives the same results as found on Figure 38 and is summarized on Table 18.

Table 18: Theoretical and practical DNA fragment sizes, in bp, after pZErO-2 restriction with the five chosen enzymes for p70 analysis.

	***Ase*I**		***Eco*RI**		***Rsa*I**	
	Theoretical	Practical	Theoretical	Practical	Theoretical	Practical
	3238	3250	3297	3300	1900	1800
	59	-			1058	1050
					279	300
					60	-
Total	*3297*	*3250*	*3297*	*3300*	*3297*	*3150*

	***Sap*I**		***Sph*I**	
	Theoretical	Practical	Theoretical	Practical
	1666	1700	2078	2150
	1421	1450	1219	1200
	210	-		
Total	*3297*	*3150*	*3297*	*3350*

Analysis of p70 restriction gives the same results as found on Figure 38 for p60 and are summarized on Table 19.

Table 19: DNA fragment sizes, in bp, after p70 restriction with *Ase*I, *Eco*RI, *Sap*I and *Sph*I.

	***Ase*I**	***Eco*RI**	***Sap*I**	***Sph*I**
	3750	3300	3500	3050
	2500	1650	1650	2450
		1500	1200	1200
Total	*6250*	*6450*	*6350*	*6700*

According to these results, it is has been assumed that recombinant plasmid p70 genetic map is the same as p60; it should be the same insert.

3.9.3 Genetic map creation of recombinant plasmid p76

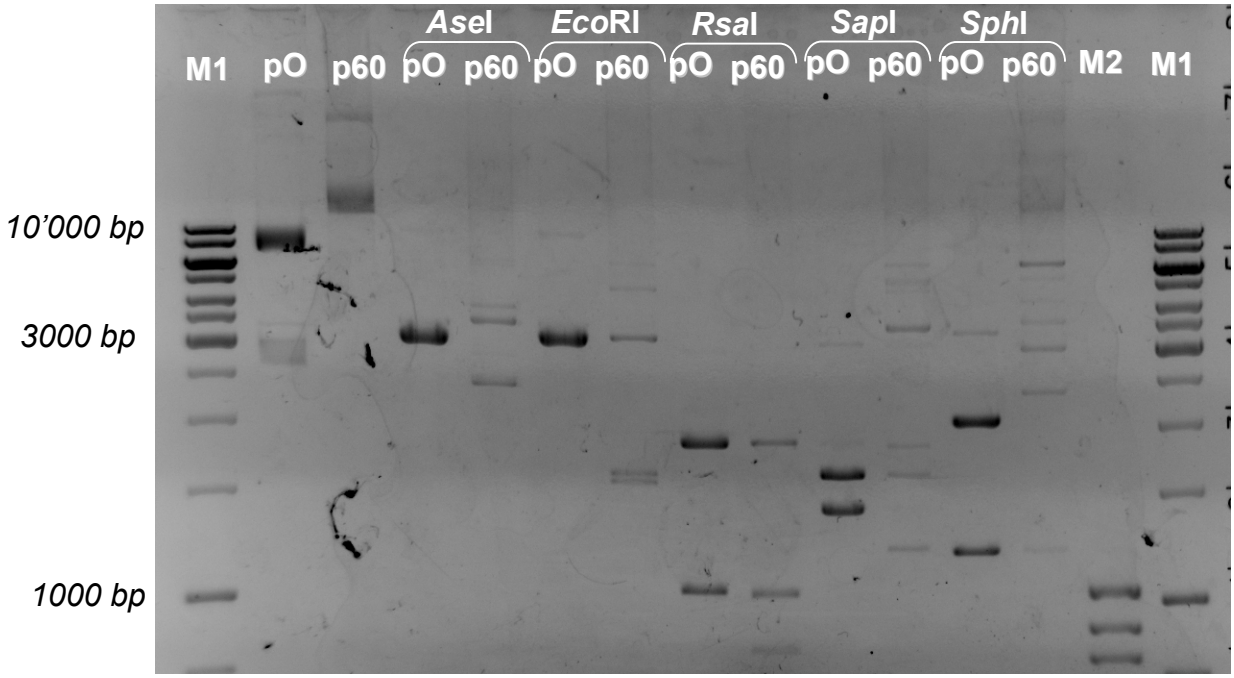


Figure 42: *Restriction of pZErO-2 vector and of recombinant plasmid p76 with the five chosen enzymes.*
M1: GeneRuler 1 kb DNA ladder (Fermentas), pO: pZErO-2 vector, p60: recombinant plasmid extracted fromm clone c60; M2: Ladder 100 bp (GeneRuler).

pZErO-2 and recombinant plasmid p76, extracted from clone 76, have been digested with the five chosen enzymes and regrouped in Figure 42. Analysis of pZErO-2 restriction gives the same results as found on Figure 38 and Figure 41 and is summarized on Table 20.

Table 20: Theoretical and practical DNA fragment sizes, in bp, after pZErO-2 restriction with the five chosen enzymes for p76 analysis.

	***Ase*I**		***Eco*RI**		***Rsa*I**	
	Theoretical	Practical	Theoretical	Practical	Theoretical	Practical
	3238	3200	3297	3100	1900	1850
	59	-			1058	1100
					279	300
					60	-
Total	*3297*	*3200*	*3297*	*3100*	*3297*	*3250*

	*Sap*I		*Sph*I	
	Theoretical	Practical	Theoretical	Practical
	1666	1700	2078	2050
	1421	1400	1219	1250
	210	-		
Total	*3297*	*3100*	*3297*	*3300*

Analysis of p76 restriction gives the same results as found on Figure 38 for p60 and is summarized on Table 21.

Table 21: DNA fragment sizes, in bp, after p70 restriction with *Ase*I, *Eco*RI, *Sap*I and *Sph*I.

	*Ase*I	*Eco*RI	*Sap*I	*Sph*I
	3500	3100	3500	3050
	2450	1650	1700	2400
		1550	1200	1200
Total	*6250*	*6450*	*6350*	*6650*

According to these results, it has been assumed that the genetic map of recombinant plasmid p76 is the same as for plasmids p60 and p70; it should be the same insert.

3.9.4 p60 insert DNA sequencing

Clone c60 extracted plasmid DNA has been sent for extern DNA sequencing using Primer Walking non-assembled service (Microsynth). The two primers used for the beginning of the DNA sequencing were SP6 and T7, present in pZErO-2 MCS (see Figure 9). After assembling, results of this sequencing are shown on Figure 43.

Insert start 1

*Eco*RI

```
>p60 insert sequence
CTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTCACGTGG     8
GTTTTTTCTTTATTCATTTTATAAACTCATAATACGCCTAAGCCCAGTTCTGTCAAGGCT    68
TTAGCCAGCTTTTTAAAAAGTCCGTTGTTTCTAATAGTATCAAGATAAGAAGAAACCGTC   128
GAAAAAACGACGGTTTCAAACCCCAAAAAGCAGATCAGCAAAAATCGCCGAACTGCTTTT   188
TAAGACCATGGCTTTCAGCCATACTGACCAGCTGAACCAGCTGGACCCCATAAATGAGCG   248
GGAGCCCCCGCTCAAACTCACCCCGCACTCGCCGTGAGGCAGGCAAAAAAAGCAGGTGTG   308
CTTTTCTTTGCATGCGCAAAAGTGTCTTGGTCTAGTGAGTCAGTCAACTCCTTAAAGCCT   368
CAAAAAGGGACTAACTGCTAAAGCAGTAAGTCCCTTTAATTCGATTTACCGTTGACTGAC   428
GGCCATTAACCAGCTTCAATGATCATCAGCGGGTAAATTAAAGCCCTAGGCCAAGTTCAA   488
AGACTTGGACCATCTAATTCATCATGCTGTCTCTCTGAACGTTCCTGTGGCCTCTGAGGG   548
GCTTTTTTAGACGGTTCTTTTAGTCCAGCTCGTTCTAGCCACTCACTTGGTAAGCCAACA   608
CCTAATTTGCGCTGTAAGAACCGGTCAACGCTCCGAACAATTCCTTGTAACGTATCCACT   668
AATTTTTGGAGCTTCTCATTTTTCTTGCTTAAATCATAATTATTGTCTTTAACTTCAAGC   728
AGTTGATAATTTAAGCTTTGATTATCAAGTTTTAAACGGTTCTGTTGTTGATTATAAATA   788
TCACTGACAGCTGCTCGAGATTCTAAGTCATGAAGATCACTTTTTCTAACAACCACTTTA   848
TCCTTGCTTAAAAGAGTAGGTTTGCTCTCAATTTCCTGCTGATCACGAGGTTTAAGTTCA   908
GCAAGGCGTTTTCTGGCATCTTCAAGTTCAGCTTGTATTTCTCGTTTTTGAAGCGTCGCC   968
TTTTTCAAGTCTTCCCGCATAGCTTTGTATTCTGGTACCGTTAAACTTTTATGTTGCGAT  1028
TCTTGAATACCACGTTCAACATCAAATCCGTGTTGTCGTAAATAAACTGGCAATTGATCT  1088
TGAACGTTTTGCAAAGCCGCACGATTAAACACCCGTTTAGCCGATAATTTATACTCATCA  1148
TCAAATGGAACAATTCCCATATGCATATGGGGCGTACTCTCATCAAGATGAACAATCGCA  1208
TAACGAATGTTTTCTTCACCAAATTTTTCAGCAAAGTAAGCTTTAGCTGTTTCAAAATAT  1268
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TTGCGCGTATCAGCCGCCGTTAAATTAGCAAAGAAATTGCTATCGCTCGAAATAATCCAT  1328
TCATTGACTAAAACGGCATCTTTTCTGACCGCTCGCTGACTGGTTTTATGCTCGTTAATA  1388
TAAGCCTCAATATCCGTTTTGAAATGGTTAGTCCGACCAGCAACTAAATCATAATTTAAG  1448
TCAGAACGGTTAACGTCAATATCAGTATTTTTGTGATGTCGGGTTCGGCGTTGATCATGA  1508
TTACCCAAGCCAACTAAATTATCAGCTTTTAATTTCTGCATATTAGCCACTAAATAACTC  1568
ATTTCACCACTCCATTCCAAACAGCTAAATTTGGTTAGAGCATAGCACAGAATTCGGAAA  1628
ACCTCCATGCAAGGTATAGCCCAATATACTTTACCTTGTAAAGTTTGGGCTCTACGAGGT  1688
AGTAGCGTTCTCCCTTCCTACTGGGAGAATTGCTATTTTTCTGCGCCTATTATCCGGCTA  1748
GAAAAACCTCCGGCGGGCTATTGCATAGCGAGCGCATTTCATGCAGAACTCTACCCGAGT  1808
GGGGCAAGCTGATGCCAGCTTAACCACAAAAAAACTATGAGAAATAAATCTCATAGTTAA  1868
ACACAACATGCTGATTGGTTACAAAATGTAAGTGTATAGAGTATTGTATAAATTCTTTTT  1928
TACTTATGTCTTTTGTTATATTTATAACTAATATAATTACCAACAACTCCAGATAGCAAA  1988
CTAACGATAAGCAATAATATTACCCAATATAAAAAAGGGTGAGTACCAAATATTTTATTC  2048
AAAAAAGGCTCAGCAAAAAGGAAAAACAAAAAAACGCAAAACGTAATAGAAAAAGGTATC  2108
CAAAATGCAGTTTTATTTTTATTTTTCATCTCTAGTCTCCTTATCTTTCAATACTTCACA  2168
AATTTTCCATCTATACGAAATAATTTTTACGCTGATAGTCCCACTTAGCAACAATTTCAC  2228
GCACTACTTGATCAGTTTCTTGATCACCACTATCCGTATTAATCAAATCGCCATTTTCAA  2288
CATCTTCTAATTGCAACTGCTTGCGAATTTCTTTAAGCAAGCCACCATAACTAATTTGCC  2348
GGGAACCAGCCAAAGCTTGTTCCAAATCATCAATTACTTGTAGATCTTGTTCTTGATTAT  2408
TAGTTAAAATATCTTTGGACTTCACCTGATATTTAGCCGTTTCTTGAGCACTAGCCAGCA  2468
AGGAATTTTTCTGGCGTTTCACATTCGGTTTAACCGCTTCAACATTCACAACCGGCGCAT  2528
AAGCTAATTTCATCGCTCGTTGCCAATATCTAGTCCATTCTGCTTGTGAAATATAATTAT  2588
CAGTTCCTGTAAAATAACTCGATTTCATAAAAAGCAAAACATGCATATGGTGGTGATACA  2648
TCGGCTGATCTGCTTCGTGATTAATGGTAACCTCAGTTGAACGCACATAGCCCAACAAAT  2708
TCTTAGCTGGCTTTTTATAACGCATTAAATCACGAATGGCTCGTCCCATTTGTCGTAATT  2768
CACTTTTTAACTGATTACCGGTGGTATTTTCAACCGTCAGCGTTAAAAATAAGAACCGAC  2828
CCGTTTTTCGCTGTTTAACTGCTTCTGTCAAAATTTGAGTTAACTGGTTAGATTGTTTCA  2888
TTGACCGCCGCCAATTACATAACGGACACAAACGGGAATGGCAAAACCAAGTCTGAGCCA  2948
ATTTTTTGTGACCATTTTTATCTTCCACAAAACGTAATACTTCACCACACTCTTTAACTC  3008
GATGGGCTTTCTTGTAGTGCAACATTTGTAAATAATCACCATACTGTAAATTCTCTAACT  3068
TATGTTCTCGCCATGGTCGTACTTTTCCCGACTGCGATCGATCAACTAAAACTTTTCTAT  3128
CAGCCAAACTAAAAAGCTCCTCTCATCCAACCACGTGAGAAGAGCTAATCTTTTATGTAT  3188
TTACTAAAACTAAGGGCTTGACGCTTACTAACTCAAGCTTTAAACTATGTCTAGTAAATA  3248
CGAATATTCAAGATTAACTCACGTGTTCCGGGTCGCCAAACTAGGAATTCTGCAGATATC  3308
CATCACACTGGCGGCCGCTCGAGCA                    EcoRI
                                             Insert end 3298
```

Figure 43: *p60 insert DNA sequence*
in brown=pZErO-2 sequence, underlined=homologous pF8801 sequence

As seen on Figure 43, p60 insert sequence is surrounded by two *Eco*RI recognition sites (in red), which were used to insert it into pZErO-2 vector (in brown). The insert size is 3298 bp. *pedA* gene is absent from p60 insert DNA sequence. Besides, similarities between p60 insert and *Pediococcus damnosus* plasmid pF8801 has been found. pF8801 region between 2085 bp and 4853 bp is homologue at 71% to p60 insert sequence (Blast). All regions underlined on Figure 43 correspond to p60 insert sequences homologous with pF8801. Region 152 bp to 227 bp and region 676 bp to 979 bp had no significant similarities with any other sequences neither with *pedA* gene. For region 1687 bp to 2185 bp, same results have been found.
*Eco*RI recognition sites surrounding p60 insert, at 1 bp and 3298 bp, are homologous to the same sequence on pF8801 situated from 3741 bp to 3746 bp. This could mean that p60 insert was circular before subcloning into pZErO-2. Besides, the three regions found on p60 insert sequence, which are not homologous to pF8801 or any other sequences could explain that p60 is in fact one of *P.damnosus* plasmid with homologous regions to pF8801.

As p60 insert did not give the expected results, a control has been performed using Dot blotting and *pedA* hybridization, shown on Figure 44, in order to confirm the presence of *pedA* gene in this recombinant plasmid p60 as well as in p70 and p76.

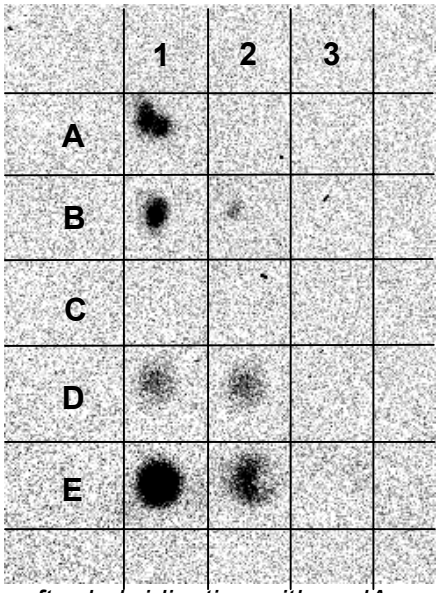

Figure 44: *Dot blot membrane after hybridization with pedA probe and CDP-star detection for p60, p70 and p76 (=control).*
A1/B1= c60 plasmid DNA; A2/B2= c70 plasmid DNA; A3/B3= c70 plasmid DNA;
D1/E1= P.damnosus genomic DNA; D2/E2=P.damnosus plasmid DNA; D3/E3= negative control.

On Figure 44, *P.damnosus* plasmid DNA extracted with Wizard Plus SV Minipreps kit (Promega) and *P.damnosus* genomic DNA extracted with NucleoSpin Tissue (Macherey-Nagel) gave positive responses. Concerning the negative controlled, composed of sterile water, ethanol and NaOH, it gave a negative response.

For the samples analyzed with this *pedA* hybridization, three different signals have been found. Plasmid DNA extracted from clone 60 with Wizard Plus SV Minipreps kit (Promega) gave a positive respond to *pedA* hybridization with high intensity; pedA gene seems to be well present in this culture. For plasmid DNA from clone 70, a real low positive response is detectable on spot B2 but not on spot A2 (samples analyzed twice). Finally, plasmid DNA from clone 76 gives no response to *pedA* hybridization.

These results seem to confirm *pedA* presence in c60 plasmid DNA and also maybe in c70 plasmid DNA. It is then assumed that each clone possesses two or more plasmids, as in a mixed culture. Recombinant plasmid p60 containing the insert similar to a region of pF8801 as well as *P.damnosus* plasmid containing pedA gene would then be both present in clone c60. However, positive response found for *pedA* hybridization is contradictory with DNA sequencing result.

4 Discussion

In order to sequence pediocin PD-1 operon, a method has previously been developed using directly *Pediococcus damnosus* genomic DNA (see Semester Work, Dumas, 2007). However, this technique did not give adequate results and identification of the genetic elements involved in the synthesis of PD-1 was therefore not possible. However, results from another project show that *pedA* gene, corresponding to PD-1 operon known sequence, could be present in one of *P.damnosus* plasmids (Ledda, 2007). This theory had then to be confirmed in to order to use only the plasmid containing *pedA* gene for this diploma work. A simpler technique can thus be developed to expand PD-1 operon sequence.

4.1 Localization of pedA *gene*

Pulsed-Field electrophoresis has been performed to separate *P.damnosus* different DNAs. Seven different bands, named α, β, γ, δ, ε, ζ and π, have been separated using this method and are visible on the agarose pulsed-field gel after UV detection (Figure 24); they correspond to the different kinds of DNA contained in *P.damnosus* bacteria. The bands near the wells are chromosomal DNA since they are large fragments that do not migrate really fast. Concerning the other bands, they certainly correspond to plasmid DNA since they are smaller fragments that migrate faster. However, DNA separation was not optimal during pulsed-field electrophoresis; DNA is still present in the wells of the gel.

DNA bands have then been transferred from the gel to a membrane by Southern blotting. A picture of the agarose gel taken after transfer shows no more visible DNA (Figure 25). After immobilized DNA on the membrane by UV, hybridization with *pedA* probe has been performed to localize which DNA band(s) contain(s) *pedA* gene. To do so, *pedA* probe had first to be created using AlkPhos Direct Labelling Kit (Amersham). This probe contains *pedA* gene and alkaline phosphatase enzyme, which uses CDP-Star® substrate to produce light. A control of the probe efficiency has been performed by immobilizing the probe on a membrane and adding CDP-Star solution; it showed intense light production after chemiluminescence exposure (data not shown). Hybridization of the membrane with *pedA* probe has been carried out and the results, after CDP-Star detection and chemiluminescence exposure, point out three distinct signals (Figure 26). The first two signals correspond to bands α and β; it is certainly one of *P.damnosus* plasmid in two different forms that did migrate at different speeds. These two bands have been respectively named plasmid α and β. The third signal shows *pedA* presence in the gel wells (band ζ), it can be explained by the fact that DNA that did not migrate is still situated in these wells and corresponds to *P.damnosus* DNA, including plasmids α and β.

4.2 Plasmids α and β extraction from the agarose gel

Following the results discussed above, bands α and β have been extracted from the agarose gel. A picture taken under UV light of the gel after excision shows that DNA bands have been well cut off (data not shown). Plasmids α and β have then been isolated from the agarose blocks and purified. This step turned out to be extremely delicate, since plasmids α and β size is about 50 kbp and such big DNA fragments are difficult to extract from agarose gel. A screening of the better extraction method had to

be performed to obtain an optimal DNA concentration after purification of the plasmids. Four different kits have been tested to extract plasmids α and β. Extremely low DNA concentration has been found for each kits. It has been decided to carry through this diploma work using plasmid α and β to see if results were possible with this method that has been named “Strategy 1: α and β plasmids extracted with NucleoSpin® Extract II kit (Macherey-Nagel)”.

4.3 Strategy 1: Plasmids α and β extracted from P.damnosus pulsed-field electrophoresis gel with NucleoSpin® Extract II kit (Macherey-Nagel).

This strategy incorporates the extraction of the plasmids α and β from the Pulsed-Field electrophoresis gel with NucleoSpin® Extract II kit (Macherey-Nagel) and their restriction with *Eco*RI and *Hind*III.

Confirmation of *pedA* gene presence on both extracted α and β plasmids has been realized using Dot Blot and *pedA* hybridization technique and shows positive results (Figure 27). The restriction of these plasmids has been performed using *Eco*RI and *Hind*III but as DNA concentration was too weak in each plasmid solution, it has not been possible to realize a control of this restriction by electrophoresis. However, control of pZErO-2 restriction with these two enzymes has been made and controlled by electrophoresis; two bands corresponding to linearized pZErO-2 after restriction with *Eco*RI and *Hind*III are detected respectively at 3400 bp and 3200 bp (Figure 28). This small difference in size between these two fragments cannot be explained; pZErO-2 digested with *Hind*III should even be longer than with *Eco*RI since the small insert present in original pZErO-2 is taken away with this enzyme.

Ligation of plasmids α and β fragments with linearized pZErO-2 vector has been realized with T4 DNA ligase and controlled by electrophoresis. The result shows no visible differences between self-ligated pZErO-2 and recombinant plasmids with inserts (Figure 29). This can be explained by supposing that self-ligated pZErO-2 is present in high quantity even in presence of insert. Recombinant plasmids, present in low concentration, are thus not detectable on the electrophoresis gel.

Insertion of these recombinant plasmids in *E.coli* TOP10 cells by electroporation gave, after screening on LB-kanamycin plates, 137 colonies for plasmid α and 63 colonies for plasmid β. Out of these colonies, 100 clones have been isolated for plasmid α and 60 clones for plasmid β. After DNA extraction of these 160 clones, regrouped in 16 series to minimize number of manipulations and time, PCR screening with primers pedF and pedR (for details, see appendices *8.4 PCR product of pedA gene with pedR and pedF primers*) has been performed; the results show only 3 positive series to *pedA* PCR-amplification (Figure 30). Unfortunately, for the further PCR screening analysis, a contamination with the plasmid containing *pedA* gene has been detected in the working place. Results using PCR screening were thus no more repeatable and this method had to be stopped. It has been replaced by Dot blotting with *pedA* hybridization. This method turned out to be more repeatable.
Using Dot blotting and *pedA* hybridization, no clones out of the 160 isolated were *pedA* positive. This strategy, using plasmids α and β, did not gave any results (Figure 31). Isolating more clones could have been a solution, but, as plasmid α and β concentrations were so weak, a second strategy, described below, has been established in order to have a better plasmid DNA concentration.

4.4 Strategy 2: Plasmid DNA extracted from P.damnosus culture with Wizard Plus SV (Promega) using EcoRI for its restriction strategy.

This strategy incorporates the extraction of plasmid DNA from *P.damnosus* culture with Wizard Plus SV kit (Promega) using *Eco*RI for its cloning strategy. Pulsed-field electrophoresis is thus not used in this method and therefore, plasmid extraction from agarose is avoided. Plasmid DNA is directly extracted from *P.damnosus* culture (Figure 32) and gives an optimal DNA concentration of about 70 µg/ml.

Confirmation of *pedA* gene in plasmid DNA has been performed using Dot blotting and *pedA* hybridization; results show a positive response to *pedA* probe (Figure 31), which means pedA gene is present in *P.damnosus* plasmid DNA. pZErO-2 hybridization with *pedA* probe gives negative response so no interaction between this vector and *pedA* probe is observable.
Restriction of plasmid DNA has been made using *Eco*RI and its insertion into linearized and dephosphorylated pZErO-2 has been performed using T4 DNA ligase. Insertion of these recombinant plasmids in *E.coli* TOP10 cells by electroporation gave, after screening on LB-kanamycin plates, 302 colonies in total. Out of these colonies, 82 clones have been isolated and analyzed by Dot blotting and *pedA* hybridization. To minimize number of manipulations and time, these 82 clones have first been regrouped in 10 series. After Dot blotting and *pedA* hybridization, 3 out of these 10 series turned out to be *pedA* positive: series 7, 8 and 9 (Figure 33). DNA extraction for each clone of these 3 series has been performed and analyzed once again via Dot blotting and *pedA* hybridization; clones 60, 70 and 76 gave positive responses to pedA hybridization (Figure 34). As pZErO-2 vector gave negative response, the interaction between pedA probe and the three different recombinant plasmids has to come from the insert.

A genetic map of these three *pedA* positive clones has been constructed using a restriction strategy described in the results part. After comparing the results to pZErO-2 theoretical restriction (Figure 35) as well as to *pedA* gene theoretical restriction (Figure 36), the genetic map of p60, recombinant plasmid of clone 60, has been established (Figure 40). Concerning p70 and p76 genetic maps, they turned out to be the same as for p60. These three recombinant plasmids are identical.

p60 insert has been sequenced using Primer Walking non-assembled and one-strand external service (Microsynth). Once these sequencing results received, an insert of 3298 bp has been assembled (Figure 43). Two *Eco*RI recognition sites surround the insert and pZErO-2 sequence is also observable at each extremity, it means that the insertion of *P.damnosus* total plasmid DNA digested with *Eco*RI did work. However, *pedA* gene has not been found into p60 insert. In addition, when analyzing p60 insert with bioinformatics tool Blast (NCBI), a high similitude with *P.damnosus* plasmid pF8801 has been established. This means that another *P.damnosus* fragment has been inserted into pZErO-2 instead of the fragment containing *pedA* gene.

According to sequencing results, it can be deduced that maybe p60 insert is one of *P.damnosus* plasmid similar to pF8801. Indeed, *Eco*RI sites situated at the start of p60 insert (1 bp) and at the end (3298 bp) do overlay and are homologous to the same pF8801 region (3741 bp to 3746 bp). p60 insert is also constituted of three regions that are not similar to pF8801. These observations could explain that this insert may be a cryptic plasmid (=which encodes no known function) linearized and inserted in pZErO-2.

Several different hypotheses can explain the reason why the wrong insert is present in pZErO-2 even though positive response to *pedA* hybriditation has been found. It can be a mixed culture with *E.coli* cells containing two different plasmids: p60 and *P.damnosus* circular plasmid in which *pedA* gene is present. In this case, the plasmid containing *pedA* gene has to have a high copy number in order to multiply in the cell. As DNA sequencing has been made via Primer Walking, only the insert situated in pZErO-2 has been recognized by the two primers T7 and SP6 and sequenced. This is why if another plasmid containing *pedA* gene was also present in extracted plasmid DNA, it was not detected while sequencing p60 insert.

Another hypothesis could be a homology between *pedA* gene and pF8801. Indeed, a specific region of pF8801 could have a homology with *pedA* probe and an interaction between these two compounds, during hybridization, would be possible. This hypothesis has been controlled using bioinformatics tool "ClustalW multiple sequence alignment", results are shown on appendices *8.12 Homology between pF8801 and pedA probe (ClustalW)*. pF8801 region situated between 3635 bp and 3789 bp possesses a homology with *pedA* probe. Indeed, a homology of 48.5 % is found but seems to be too low to explain a real interaction between these two compounds after washing steps.

It is then assumed that the problem related to the insert similar to a pF8801 region came from a mixed culture. This hypothesis can be supported by the results given by the control carried out via pedA hybridization for clones c60, c70 and c76 plasmid DNA (Figure 44). Plasmid DNA from c60 gives once again positive response; it seems that *pedA* is well present in this clone since two analyzes confirmed it (Figure 34 and Figure 44). However, concerning c70 and c76, the control did not give positive response or only a very low signal for c70 plasmid DNA (Figure 44). No logical reason has been found for this.

5 Conclusion and perspectives

Four different aims have been pointed out for this diploma work. The first goal was to separate *P.damnosus* DNA on pulsed-field electrophoresis gel, to confirm that *pedA* gene is present on one of *P.damnosus* plasmid and to extract this plasmid. This has been done and *pedA* gene has been confirmed in plasmid α or β, certainly the same plasmid but in two different forms. However, extraction of these two plasmids from agarose gel has been a critical step and gave really weak DNA concentration in plasmids solutions. In spite of using different extraction kits, DNA concentration could not be increased.

The second objective was to construct a plasmid library using different restriction enzymes and by subcloning the fragments into vector pZErO-2. One plasmid library of 160 clones has been constructed for plasmids α and β digested with *Eco*RI and *Hind*III and inserted into pZErO-2.

For the third goal, an identification of recombinant plasmids containing *pedA* gene either by PCR screening or by hybridization with *pedA* probe and localization of *pedA* region in the insert had to be done. Using PCR screening, results turned out to be no repeatable on account of *pedA* gene-containing plasmid contamination in the working place. Indeed, by dint of working always with the same plasmid, a contamination was observed

and therefore, results could not be taken into consideration. Dot blotting followed by *pedA* hybridization has been preferred. Analyzing the 160 clones showed no clones positive to *pedA* probe. Hypothesis for lack of result could be the weak DNA concentration noticed in plasmids α and β solutions after extraction from the agarose gel.

Therefore, a second strategy has been developed using directly plasmid DNA extracted from *P.damnosus*. Two plasmid libraries have been constructed with respectively *Eco*RI and *Hind*III using *P.damnosus* total plasmid DNA. The first library, using *Eco*RI for restriction, was composed of 82 clones. Dot blotting and *pedA* hybridization analysis gave three *pedA* positive clones, which were named c60, c70 and c76. Out of these three clones, plasmid DNA has been extracted and digested using five different restriction enzymes, *Ase*I, *Eco*RI, *Rsa*I, *Sap*I and *Sph*I in order to construct their genetic maps. Results show the same insert for the three analyzed plasmids, named respectively p60, p70 and p76. According to these restriction results, the insert size was estimated around 3 kbp.

Finally, the fourth objective was to sequence operon PD-1 missing regions and to identify the Opening Reading Frame. External DNA sequencing by Primer Walking (Microsynth) of p60 insert has then been performed to expand PD-1 known sequence. Unfortunately, sequencing results show an insert without *pedA* gene and, instead, composed of a region of *P.damnosus* plasmid pF8801 situated between 2085 bp and 4853 bp. Two hypotheses have been pointed out to explain this: it is either a mixed culture composed of two plasmids, p60 and *P.damnosus* plasmid that contains *pedA* gene, or a homology between a region of the insert and *pedA* probe. The first hypothesis seems to be more suitable since homology found between *pedA* probe and pF8801 is too low to have a real interaction during hybridization.

To conclude, three out of the four aims seen above have been achieved. The plasmid library from *P.damnosus* plasmid DNA using *Eco*RI has been stocked for further analysis. In addition, a second plasmid library using *Hind*III of 63 clones has been constructed and stocked in order to possess even more possibilities to find *pedA* positive clone. Because of a lack of time, this library has not been analyzed by *pedA* hybridization.

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8 Appendices

8.1 Illustrations table

8.2 pedA PCR-amplification Primers

Ped F (Microsynth)

5' CCGCAGAAGAATCATCAGGT 3'

- Length = 20 nucleotides
- Tm = 52°C
- %GC = 50

Ped R (Microsynth)

5' CATGTTGCCAAATGATCACA 3'

- Length = 20 nucleotides
- Tm = 48°C
- %GC = 40

8.3 pedA sequence with primer pedR and pedF

```
   1 ATAGCCATAT TTCATCTTTT TATCTCCTTA CTTATGTCAT TAAGTAATGA CACGGTTCTA
  61 ACCCTTTAAT TATACAGTAT CAAAAAAGAG GCCACACTGT TAAGATGTGA CACAATCATT
 122 AAAATTTGAT TTTTATTAAG AATAGTATAC GAAACATTGA AGGCTCAAAA ATTTTCTGGA
 182 TTCCCCTCAA TTTAATCCAC TTTTACCATA TAACTTGTTT ACTTCACTTG AAAGGGAATA
 242 TTTGGAACTA AAATGAAATT GTTCACAAAT ATGCAGATAG GAGGATTATT TATAGTTTGA
 302 CAGCCCTAAT CACAAAGTGT ACCTCATTTA CAATCTGAAA AGGAGACGTG AGTTGTTATA
                   Primer F
 362 GAAAAATTTA ATGAAGTCCG CAGAAGAATC ATCAGGTAAT GTTTTGGAAG AATTAAATAA
                                    Primer R
 422 TGCAGCTTGG TATGATTAGT GGTGGTAAGA AGATCAAGAA GAGTTCTAGT GGTGACATTT
 482 ACCTTAACAA GTGAATGTGA TCATTTGGCA ACATGGGTAT GTTGCTAAAA ATCATTTTGA
 542 CTATTTTTTG CTATTAATAA GAGTGATTAG GGTTGCTTCA AGCTATAAAT TCTAATGATC
 602 ACATGGTAAA AAAAACAGAT TCCGTGCAGG GCCAAATTGA AAAAGCAATA GGGAGATGTA
 662 TTACAGCTGG CTATGGATAA TTTAAGTAAG TTC
```

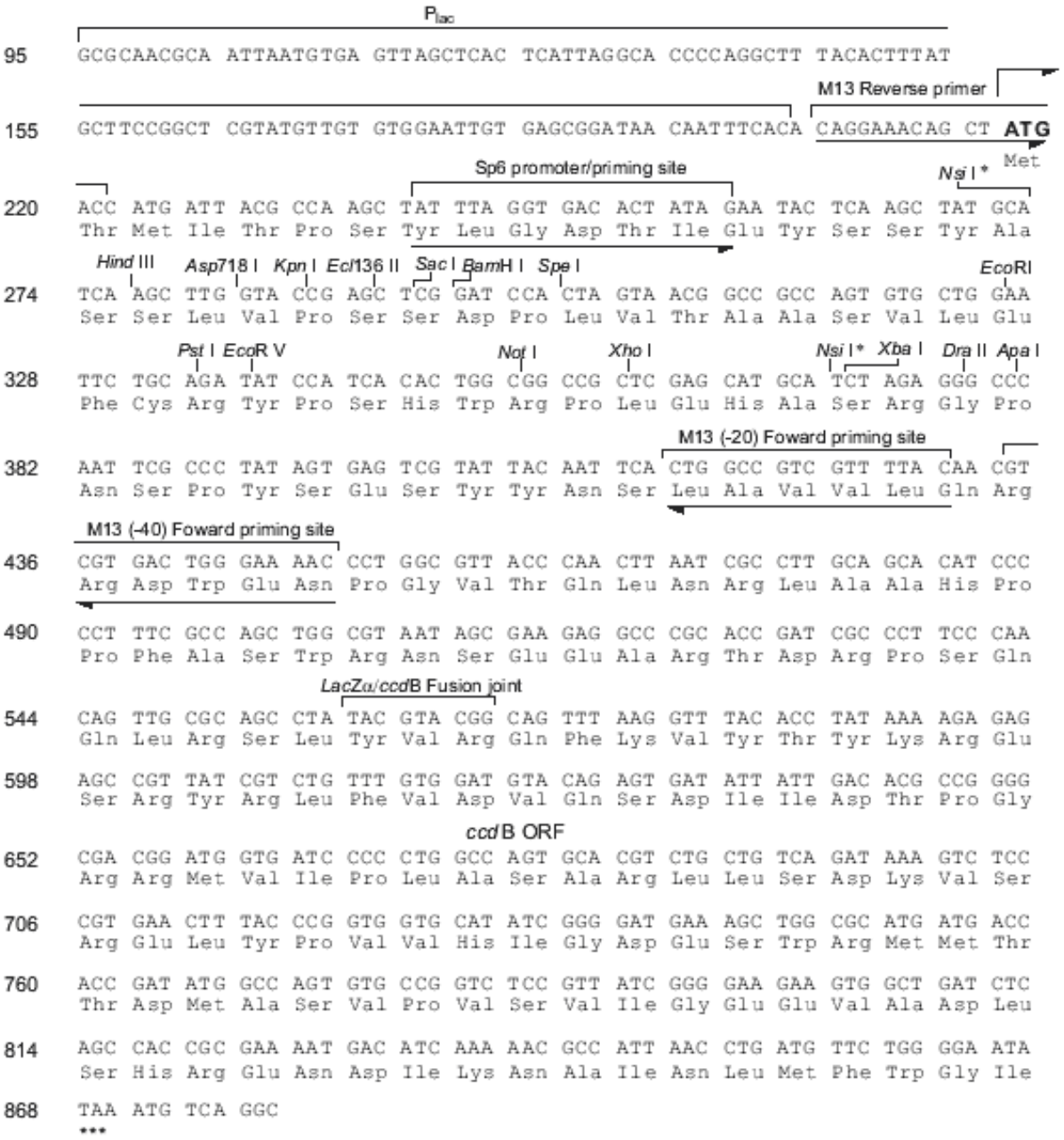
8.4 PCR product of pedA gene with pedR and pedF primers

```
1   CCGCAGAAGA ATCATCAGGT AATGTTTTGG AAGAATTAAA TAATGCAGCT TGGTATGAT

61  GTGGTGGTAA GAAGATCAAG AAGAGTTCTA GTGGTGACAT TTACCTTAAC AAGTGAATG

121 TGATCATTTG GCAACATG
```

8.5 Detail of pZErO-2



*The two *Nsi* I sites in the polylinker are the only *Nsi* I sites in the vector.

8.6 *pZErO™-2 sequence*

```
   1 agcgcccaat acgcaaaccg cctctccccg cgcgttggcc gattcattaa tgcagctggc
  61 acgacaggtt tcccgactgg aaagcgggca gtgagcgcaa cgcaattaat gtgagttagc
 121 tcactcatta ggcaccccag gctttacact ttatgcttcc ggctcgtatg ttgtgtggaa
 181 ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagctat
 241 ttaggtgaca ctatagaata ctcaagctat gcatcaagct tggtaccgag ctcggatcca
 301 ctagtaacgg ccgccagtgt gctggaattc tgcagatatc catcacactg gcggccgctc
 361 gagcatgcat ctagagggcc caattcgccc tatagtgagt cgtattacaa ttcactggcc
 421 gtcgttttac aacgtcgtga ctgggaaaac cctggcgtta cccaacttaa tcgccttgca
 481 gcacatcccc ctttcgccag ctggcgtaat agcgaagagg cccgcaccga tcgcccttcc
 541 caacagttgc gcagcctata cgtacggcag tttaaggttt acacctataa aagagagagc
 601 cgttatcgtc tgtttgtgga tgtacagagt gatattattg acacgccggg gcgacggatg
 661 gtgatccccc tggccagtgc acgtctgctg tcagataaag tctcccgtga actttacccg
 721 gtggtgcata tcggggatga aagctggcgc atgatgacca ccgatatggc cagtgtgccg
 781 gtctccgtta tcggggaaga agtggctgat ctcagccacc gcgaaaatga catcaaaaac
 841 gccattaacc tgatgttctg gggaatataa atgtcaggcc tgaatggcga atggacgcgc
 901 cctgtagcgg cgcattaagc gcgcgggtgt ggtggttacg cgcagcgtga ccgctacact
 961 tgccagcgcc ctagcgcccg ctcctttcgc tttcttccct tcctttctcg ccacgttcgc
1021 cggctttccc cgtcaagctc taaatcgggg gctcccttta gggttccgat ttagagcttt
1081 acggcacctc gaccgcaaaa aacttgattt gggtgatggt tcacgtagtg ggccatcgcc
1141 ctgatagacg gtttttcgcc ctttgacgtt ggagtccacg ttctttaata gtggactctt
1201 gttccaaact ggaacaacac tcaaccctat cgcggtctat tcttttgatt tataagggat
1261 gttgccgatt tcggcctatt ggttaaaaaa tgagctgatt taacaaaaat tttaacaaaa
1321 ttcagaagaa ctcgtcaaga aggcgataga aggcgatgcg ctgcgaatcg ggagcggcga
1381 taccgtaaag cacgaggaag cggtcagccc attcgccgcc aagctcttca gcaatatcac
1441 gggtagccaa cgctatgtcc tgatagcggt ccgccacacc cagccggcca cagtcgatga
1501 atccagaaaa gcggccattt tccaccatga tattcggcaa gcaggcatcg ccatgggtca
1561 cgacgagatc ctcgccgtcg ggcatgctcg ccttgagcct ggcgaacagt tcggctggcg
1621 cgagcccctg atgctcttcg tccagatcat cctgatcgac aagaccggct tccatccgag
1681 tacgtgctcg ctcgatgcga tgtttcgctt ggtggtcgaa tgggcaggta gccggatcaa
1741 gcgtatgcag ccgccgcatt gcatcagcca tgatggatac tttctcggca ggagcaaggt
1801 gagatgacag gagatcctgc cccggcactt cgcccaatag cagccagtcc cttcccgctt
1861 cagtgacaac gtcgagcaca gctgcgcaag gaacgcccgt cgtggccagc cacgatagcc
1921 gcgctgcctc gtcttgcagt tcattcaggg caccggacag gtcggtcttg acaaaaagaa
1981 ccgggcgccc ctgcgctgac agccggaaca cggcggcatc agagcagccg attgtctgtt
2041 gtgcccagtc atagccgaat agcctctcca cccaagcggc cggagaacct gcgtgcaatc
2101 catcttgttc aatcatgcga aacgatcctc atcctgtctc ttgatcagat cttgatcccc
2161 tgcgccatca gatccttggc ggcgagaaag ccatccagtt tactttgcag ggcttcccaa
2221 ccttaccaga gggcgcccca gctggcaatt ccggttcgct tgctgtccat aaaaccgccc
2281 agtctagcta tcgccatgta agcccactgc aagctacctg ctttctcttt gcgcttgcgt
2341 tttcccttgt ccagatagcc cagtagctga cattcatccg gggtcagcac cgtttctgcg
2401 gactggcttt ctacgtgaaa aggatctagg tgaagatcct ttttgataat ctcatgacca
2461 aaatccctta acgtgagttt tcgttccact gagcgtcaga ccccgtagaa aagatcaaag
2521 gatcttcttg agatcctttt tttctgcgcg taatctgctg cttgcaaaca aaaaaaccac
2581 cgctaccagc ggtggtttgt ttgccggatc aagagctacc aactcttttt ccgaaggtaa
2641 ctggcttcag cagagcgcag ataccaaata ctgtccttct agtgtagccg tagttaggcc
2701 accacttcaa gaactctgta gcaccgccta catacctcgc tctgctaatc ctgttaccag
2761 tggctgctgc cagtggcgat aagtcgtgtc ttaccgggtt ggactcaaga cgatagttac
2821 cggataaggc gcagcggtcg ggctgaacgg ggggttcgtg cacacagccc agcttggagc
2881 gaacgaccta caccgaactg agatacctac agcgtgagct atgagaaagc gccacgcttc
2941 ccgaagggag aaaggcggac aggtatccgg taagcggcag ggtcggaaca ggagagcgca
3001 cgagggagct tccagggggа aacgcctggt atctttatag tcctgtcggg tttcgccacc
3061 tctgacttga gcgtcgattt ttgtgatgct cgtcaggggg gcggagccta tggaaaaacg
3121 ccagcaacgc ggcctttttа cggttcctgg gcttttgctg gccttttgct cacatgttct
3181 ttcctgcgtt atcccctgat tctgtggata accgtattac cgcctttgag tgagctgata
3241 ccgctcgccg cagccgaacg accgagcgca gcgagtcagt gagcgaggaa gcggaag
```

8.7 *pZErO™-2 sequence with enzymes recognition sites for restriction map*

```
                                                                  AseI
   1 agcgcccaat acgcaaaccg cctctccccg cgcgttggcc gattcattaa tgcagctggc
                                                              AseI
  61 acgacaggtt tcccgactgg aaagcgggca gtgagcgcaa cgcaattaat gtgagttagc
 121 tcactcatta ggcaccccag gctttacact ttatgcttcc ggctcgtatg ttgtgtggaa
 181 ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagctat
                                                          RsaI
 241 ttaggtgaca ctatagaata ctcaagctat gcatcaagct tggtaccgag ctcggatcca
                                  EcoRI
 301 ctagtaacgg ccgccagtgt gctggaattc tgcagatatc catcacactg gcggccgctc  MCS
      SphI
 361 gagcatgcat ctagagggcc caattcgccc tatagtgagt cgtattacaa ttcactggcc

 421 gtcgttttac aacgtcgtga ctgggaaaac cctggcgtta cccaacttaa tcgccttgca
 481 gcacatcccc ctttcgccag ctggcgtaat agcgaagagg cccgcaccga tcgcccttcc
                              RsaI
 541 caacagttgc gcagcctata cgtacggcag tttaaggttt acacctataa aagagagagc
                              RsaI
 601 cgttatcgtc tgtttgtgga tgtacagagt gatattattg acacgccggg gcgacggatg
 661 gtgatccccc tggccagtgc acgtctgctg tcagataaag tctcccgtga actttacccg
 721 gtggtgcata tcggggatga aagctggcgc atgatgacca ccgatatggc cagtgtgccg
 781 gtctccgtta tcggggaaga agtggctgat ctcagccacc gcgaaaatga catcaaaaac
 841 gccattaacc tgatgttctg gggaatataa atgtcaggcc tgaatggcga atggacgcgc
 901 cctgtagcgg cgcattaagc gcgcgggtgt ggtggttacg cgcagcgtga ccgctacact
 961 tgccagcgcc ctagcgcccg ctcctttcgc tttcttccct tcctttctcg ccacgttcgc
1021 cggctttccc cgtcaagctc taaatcgggg gctcccttta gggttccgat ttagagcttt
1081 acggcacctc gaccgcaaaa aacttgattt gggtgatggt tcacgtagtg ggccatcgcc
1141 ctgatagacg gtttttcgcc ctttgacgtt ggagtccacg ttctttaata gtggactctt
1201 gttccaaact ggaacaacac tcaaccctat cgcggtctat tcttttgatt tataagggat
1261 gttgccgatt tcggcctatt ggttaaaaaa tgagctgatt taacaaaaat tttaacaaaa
1321 ttcagaagaa ctcgtcaaga aggcgataga aggcgatgcg ctgcgaatcg ggagcggcga
                                                          SapI
1381 taccgtaaag cacgaggaag cggtcagccc attcgccgcc aagctcttca gcaatatcac
1441 gggtagccaa cgctatgtcc tgatagcggt ccgccacacc cagccggcca cagtcgatga
1501 atccagaaaa gcggccattt tccaccatga tattcggcaa gcaggcatcg ccatgggtca
                              SphI
1561 cgacgagatc ctcgccgtcg ggcatgctcg ccttgagcct ggcgaacagt tcggctggcg
                        SapI
1621 cgagcccctg atgctcttcg tccagatcat cctgatcgac aagaccggct tccatccgag
     RsaI
1681 tacgtgctcg ctcgatgcga tgtttcgctt ggtggtcgaa tgggcaggta gccggatcaa
1741 gcgtatgcag ccgccgcatt gcatcagcca tgatggatac tttctcggca ggagcaaggt
1801 gagatgacag gagatcctgc cccggcactt cgcccaatag cagccagtcc cttcccgctt
1861 cagtgacaac gtcgagcaca gctgcgcaag gaacgcccgt cgtggccagc cacgatagcc
1921 gcgctgcctc gtcttgcagt tcattcaggg caccggacag gtcggtcttg acaaaaagaa
1981 ccgggcgccc ctgcgctgac agccggaaca cggcggcatc agagcagccg attgtctgtt
2041 gtgcccagtc atagccgaat agcctctcca cccaagcggc cggagaacct gcgtgcaatc
2101 catcttgttc aatcatgcga aacgatcctc atcctgtctc ttgatcagat cttgatcccc
2161 tgcgccatca gatccttggc ggcgagaaag ccatccagtt tactttgcag ggcttcccaa
2221 ccttaccaga gggcgcccca gctggcaatt ccggttcgct tgctgtccat aaaaccgccc
2281 agtctagcta tcgccatgta agcccactgc aagctacctg ctttctcttt gcgcttgcgt
2341 tttcccttgt ccagatagcc cagtagctga cattcatccg gggtcagcac cgtttctgcg
2401 gactggcttt ctacgtgaaa aggatctagg tgaagatcct ttttgataat ctcatgacca
2461 aaatccctta acgtgagttt tcgttccact gagcgtcaga ccccgtagaa aagatcaaag
2521 gatcttcttg agatcctttt tttctgcgcg taatctgctg cttgcaaaca aaaaaaccac
2581 cgctaccagc ggtggtttgt ttgccggatc aagagctacc aactcttttt ccgaaggtaa
2641 ctggcttcag cagagcgcag ataccaaata ctgtccttct agtgtagccg tagttaggcc
2701 accacttcaa gaactctgta gcaccgccta catacctcgc tctgctaatc ctgttaccag
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2761 tggctgctgc cagtggcgat aagtcgtgtc ttaccgggtt ggactcaaga cgatagttac
2821 cggataaggc gcagcggtcg ggctgaacgg ggggttcgtg cacacagccc agcttggagc
2881 gaacgaccta caccgaactg agatacctac agcgtgagct atgagaaagc gccacgcttc
2941 ccgaagggag aaaggcggac aggtatccgg taagcggcag ggtcggaaca ggagagcgca
3001 cgagggagct tccagggga aacgcctggt atctttatag tcctgtcggg tttcgccacc
3061 tctgacttga gcgtcgattt ttgtgatgct cgtcaggggg gcggagccta tggaaaaacg
3121 ccagcaacgc ggccttttta cggttcctgg gcttttgctg gccttttgct cacatgttct
3181 ttcctgcgtt atcccctgat tctgtggata accgtattac cgcctttgag tgagctgata
3241 ccgctcgccg cagccgaacg accgagcgca gcgagtcagt gagcgaggaa gcggaag
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8.8 *Plasmid library using EcoRI*

Box containing *Eco*RI plasmid library is stocked at –70°C and is composed as described above. Clones who are absent from this box means isolation step did not work.

Box Name: *Eco*RI clones
Composition of tube: 1 ml of LB-kanamycin medium + 1 ml of glycerol 87%
Number of clones: 82
pedA positive clones: 3 *clone in orange*

	1	2	3	4	5	6	7	8	9	10
A	1 *Eco*RI	12 *Eco*RI	22 *Eco*RI	33 *Eco*RI	44 *Eco*RI	55 *Eco*RI	73 *Eco*RI	87 *Eco*RI		
B	2 *Eco*RI	13 *Eco*RI	23 *Eco*RI	34 *Eco*RI	45 *Eco*RI	56 *Eco*RI	74 *Eco*RI	89 *Eco*RI		
C	3 *Eco*RI	14 *Eco*RI	24 *Eco*RI	35 *Eco*RI	46 *Eco*RI	58 *Eco*RI	76 *Eco*RI	K^+		
D	4 *Eco*RI	15 *Eco*RI	25 *Eco*RI	37 *Eco*RI	47 *Eco*RI	60 *Eco*RI	78 *Eco*RI			
E	5 *Eco*RI	16 *Eco*RI	26 *Eco*RI	38 *Eco*RI	48 *Eco*RI	64 *Eco*RI	81 *Eco*RI			
F	6 *Eco*RI	17 *Eco*RI	27 *Eco*RI	39 *Eco*RI	50 *Eco*RI	66 *Eco*RI	82 *Eco*RI			
G	8 *Eco*RI	18 *Eco*RI	28 *Eco*RI	40 *Eco*RI	51 *Eco*RI	69 *Eco*RI	83 *Eco*RI			
H	9 *Eco*RI	19 *Eco*RI	29 *Eco*RI	41 *Eco*RI	52 *Eco*RI	70 *Eco*RI	84 *Eco*RI			
I	10 *Eco*RI	20 *Eco*RI	30 *Eco*RI	42 *Eco*RI	53 *Eco*RI	71 *Eco*RI	85 *Eco*RI			
J	11 *Eco*RI	21 *Eco*RI	32 *Eco*RI	43 *Eco*RI	54 *Eco*RI	72 *Eco*RI	86 *Eco*RI			

8.9 *Plasmid library using HindIII*

Box containing *Hind*III plasmid library is stocked at –70°C and is composed as described above. Clones who are absent from this box means isolation step did not work.

Box Name: *Hind*III clones
Composition of tube: 0.8 ml of LB-kanamycin medium + 0.8 ml of glycerol 87%
Number of clones: 63
pedA positive clones: not yet analysed

	1	2	3	4	5	6	7	8	9	10
A	1 *Hind*III	11 *Hind*III	24 *Hind*III	35 *Hind*III	46 *Hind*III	57 *Hind*III				
B	2 *Hind*III	12 *Hind*III	26 *Hind*III	36 *Hind*III	47 *Hind*III	58 *Hind*III				
C	3 *Hind*III	13 *Hind*III	27 *Hind*III	37 *Hind*III	48 *Hind*III	59 *Hind*III				
D	4 *Hind*III	14 *Hind*III	28 *Hind*III	38 *Hind*III	49 *Hind*III	K^+				
E	5 *Hind*III	15 *Hind*III	29 *Hind*III	39 *Hind*III	50 *Hind*III					
F	6 *Hind*III	17 *Hind*III	30 *Hind*III	41 *Hind*III	52 *Hind*III					
G	7 *Hind*III	18 *Hind*III	31 *Hind*III	42 *Hind*III	53 *Hind*III					
H	8 *Hind*III	19 *Hind*III	32 *Hind*III	43 *Hind*III	54 *Hind*III					
I	9 *Hind*III	21*Hind*III	33 *Hind*III	44 *Hind*III	55 *Hind*III					
J	10 *Hind*III	22 *Hind*III	34 *Hind*III	45 *Hind*III	56 *Hind*III					

8.10 Insert p60, non-assembled Primer Walking, one strand (Microsynth)

Insert Pediocin SP6, non-assembled Primer Walking, one strand (Microsynth)

CTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTCACGTGGGTTTT
TTCTTTATTCATTTTATAAACTCATAATACGCCTAAGCCCAGTTCTGTCAAGGCTTTAGCCAGCT
TTTTAAAAAGTCCGTTGTTTCTAATAGTATCAAGATAAGAAGAAACCGTCGAAAAAACGACGGTT
TCAAACCCCAAAAAGCAGATCAGCAAAAATCGCCGAACTGCTTTTTAAGACCATGGCTTTCAGCC
ATACTGACCAGCTGAACCAGCTGGACCCCATAAATGAGCGGGAGCCCCCGCTCAAACTCACCCCG
CACTCGCCGTGAGGCAGGCAAAAAAAGCAGGTGTGCTTTTCTTTGCATGCGCAAAAGTGTCTTGG
TCTAGTGAGTCAGTCAACTCCTTAAAGCCTCAAAAAGGGACTAACTGCTAAAGCAGTAAGTCCCT
TTAATTCGATTTACCGTTGACTGACGGCCATTAACCAGCTTCAATGATCATCAGCGGGTAAATTA
AAGCCCTAGGCCAAGTTCAAAGACTTGGACCATCTAATTCATCATGCTGTCTCTCTGAACGTTCC
TGTGGCCTCTGAGGGGCTTTTTTAGACGGTTCTTTTAGTCCAGCTCGTTCTAGCCACTCACTTGG
TAAGCCAACACCTAATTTGCGCTGTAAGAACCGGTCAACGCTCCGAACAATTCCTTGTAACGTAT
CCACTAATTTTTGGAGCTTCTCATTTTTCTTGCTTAAATCATAATTATTGTCTTTAACTTCAAGC
AGTTGATAATTTAAGCTTTGATTATCAAGTTTTAAACGGTTCTG

Insert Pediocin SP6.1, non-assembled Primer Walking, one strand (Microsynth)

CCGAACAATTCCTTGTAACGTATCCACTAATTTTTGGAGCTTCTCATTTTTCTTGCTTAAATCAT
AATTATTGTCTTTAACTTCAAGCAGTTGATAATTTAAGCTTTGATTATCAAGTTTTAAACGGTTC
TGTTGTTGATTATAAATATCACTGACAGCTGCTCGAGATTCTAAGTCATGAAGATCACTTTTTCT
AACAACCACTTTATCCTTGCTTAAAAGAGTAGGTTTGCTCTCAATTTCCTGCTGATCACGAGGTT
TAAGTTCAGCAAGGCGTTTTCTGGCATCTTCAAGTTCAGCTTGTATTTCTCGTTTTTGAAGCGTC
GCCTTTTTCAAGTCTTCCCGCATAGCTTTGTATTCTGGTACCGTTAAACTTTTATGTTGCGATTC
TTGAATACCACGTTCAACATCAAATCCGTGTTGTCGTAAATAAACTGGCAATTGATCTTGAACGT
TTTGCAAAGCCGCACGATTAAACACCCGTTTAGCCGATAATTTATACTCATCATCAAATGGAACA
ATTCCCATATGCATATGGGGCGTACTCTCATCAAGATGAACAATCGCATAACGAATGTTTTCTTC
ACCAAATTTTTCAGCAAAGTAAGCTTTAGCTGTTTCAAAATATTTGCGCGTATCAGCCGCCGTTA
AATTAGCAAAGAAATTGCTATCGCTCGAAATAATCCATTCATTGACTAAAACGGCATCTTTTCTG
ACCGCTCGCTGACTGGTTTTATGCTCGTTAATATAAGCCTCAATATCCGTTTTGAAATGGTTAGT
CCGACCAGCAACTAAATCATAATTTA

Insert Pediocin SP6.2, non-assembled Primer Walking, one strand (Microsynth)

CTNTTTTNTGNNGCTCGCTGMTGGTTTTATGCTCGTTAATATAAGCCTCAATATCCGTTTTGAAA
TGGTTAGTCCGACCAGCAACTAAATCATAATTTAAGTCAGAACGGTTAACGTCAATATCAGTATT
TTTGTGATGTCGGGTTCGGCGTTGATCATGATTACCCAAGCCAACTAAATTATCAGCTTTTAATT
TCTGCATATTAGCCACTAAATAACTCATTTCACCACTCCATTCCAAACAGCTAAATTTGGTTAGA
GCATAGCACAGAATTCGGAAAACCTCCATGCAAGGTATAGCCCAATATACTTTACCTTGTAAAGT
TTGGGCTCTACGAGGTAGTAGCGTTCTCCCTTCCTACTGGGAGAATTGCTATTTTTCTGCGCCTA
TTATCCGGCTAGAAAAACCTCCGGCGGGCTATTGCATAGCGAGCGCATTTCATGCAGAACTCTAC
CCGAGTGGGGCAAGCTGATGCCAGCTTAACCACAAAAAAACTATGAGAAATAAATCTCATAGTTA
AACACAACATGCTGATTGGTTACAAAATGTAAGTGTATAGAGTATTGTATAAATTCTTTTTTACT
TATGTCTTTTGTTATATTTATAACTAATATAATTACCAACAACTCCAGATAGCAAACTAACGATA
AGCAATAATATTACCCAATATAAAAAAGGGTGAGTACCAAATATTTTATTCAAAAAAGGCTCAGC
AAAAAGGAAAAACAAAAAAACGCAAAACGTAATAGAAAAAGGTATCCAAAATGCAGTTTTATTTT
TATTTTTCATCTCTAGTCTCCTTATCTTTCAATACTTCACAAATTTTCCATCTATACGAAATAAT
TTTTACGCTGATAGTCCCACTTAGCAACAATTTCACGCACTACTTGATCAGTTTCTTGA

Insert Pediocin T7, non-assembled Primer Walking, one strand (Microsynth)

TGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCCTAGTTTGGCGACCCGGAACACGT
GAGTTAATCTTGAATATTCGTATTTACTAGACATAGTTTAAAGCTTGAGTTAGTAAGCGTCAAGC
CCTTAGTTTTAGTAAATACATAAAAGATTAGCTCTTCTCACGTGGTTGGATGAGAGGAGCTTTTT
AGTTTGGCTGATAGAAAAGTTTTAGTTGATCGATCGCAGTCGGGAAAAGTACGACCATGGCGAGA
ACATAAGTTAGAGAATTTACAGTATGGTGATTATTTACAAATGTTGCACTACAAGAAAGCCCATC
GAGTTAAAGAGTGTGGTGAAGTATTACGTTTTGTGGAAGATAAAAATGGTCACAAAAAATTGGCT
CAGACTTGGTTTTGCCATTCCCGTTTGTGTCCGTTATGTAATTGGCGGCGGTCAATGAAACAATC
TAACCAGTTAACTCAAATTTTGACAGAAGCAGTTAAACAGCGAAAAACGGGTCGGTTCTTATTTT
TAACGCTGACGGTTGAAAATACCACCGGTAATCAGTTAAAAAGTGAATTACGACAAATGGGACGA
GCCATTCGTGATTTAATGCGTTATAAAAAGCCAGCTAAGAATTTGTTGGGCTATGTGCGTTCAAC
TGAGGTTACCATTAATCACGAAGCAGATCAGCCGATGTATCACCACCATATGCATGTTTTGCTTT
TTATGAAATCGAGTTATTTTACAGGAACTGATAATTATATTTCACAAGCAGAATGGACTAGATAT
TGGCAACGAGCGATGAAATTAGCTTATGCGCCGGTTGTGAATGTTGAAGCGGTTAAACCGAATGT
GAAACGCCAG

Insert Pediocin T7.1, non-assembled Primer Walking, one strand (Microsynth)

CCATATGCATGTTTTGCTTTTTATGAAATCGAGTTATTTTACAGGAACTGATAATTATATTTCAC
AAGCAGAATGGACTAGATATTGGCAACGAGCGATGAAATTAGCTTATGCGCCGGTTGTGAATGTT
GAAGCGGTTAAACCGAATGTGAAACGCCAGAAAAATTCCTTGCTGGCTAGTGCTCAAGAAACGGC
TAAATATCAGGTGAAGTCCAAAGATATTTTAACTAATAATCAAGAACAAGATCTACAAGTAATTG
ATGATTTGGAACAAGCTTTGGCTGGTTCCCGGCAAATTAGTTATGGTGGCTTGCTTAAAGAAATT
CGCAAGCAGTTGCAATTAGAAGATGTTGAAAATGGCGATTTGATTAATACGGATAGTGGTGATCA
AGAAACTGATCAAGTAGTGCGTGAAATTGTTGCTAAGTGGGACTATCAGCGTAAAAATTATTTCG
TATAGATGGAAAATTTGTGAAGTATTGAAAGATAAGGAGACTAGAGATGAAAAATAAAAATAAAA
CTGCATTTTGGATACCTTTTTCTATTACGTTTTGCGTTTTTTTGTTTTTCCTTTTTGCTGAGCCT
TTTTTGAATAAAATATTTGGTACTCACCCTTTTTTATATTGGGTAATATTATTGCTTATCGTTAG
TTTGCTATCTGGAGTTGTTGGTAATTATATTAGTTATAAATATAACAAAAGACATAAGTAAAAAA
GAATTTATACAATACTCTATACACTTACATT

Insert Pediocin T7.2, non-assembled Primer Walking, one strand (Microsynth)

TATNGTNTAAATATAACAAAAGANATAAGTAAAAAAGAATTTATACAATACTCTATACACTTACA
TTTTGTAACMATCAGCATGTTGTGTTTAACTATGAGATTTATTTCTCATAGTTTTTTTGTGGTTA
AGCTGGCATCAGCTTGCCCCACTCGGGTAGAGTTCTGCATGAAATGCGCTCGCTATGCAATAGCC
CGCCGGAGGTTTTTCTAGCCGGATAATAGGCGCAGAAAAATAGCAATTCTCCCAGTAGGAAGGGA
GAACGCTACTACCTCGTAGAGCCCAAACTTTACAAGGTAAAGTATATTGGGCTATACCTTGCATG
GAGGTTTTCCGAATTCTGTGCTATGCTCTAACCAAATTTAGCTGTTTGGAATGGAGTGGTGAAAT
GAGTTATTTAGTGGCTAATATGCAGAAATTAAAAGCTGATAATTTAGTTGGCTTGGGTAATCATG
ATCAACGCCGAACCCGACATCACAAAAATACTGATATTGACGTTAACCGTTCTGACTTAAATTAT
GATTTAGTTGCTGGTCGGACTAACCATTTCAAAACGGATATTGAGGCTTATATTAACGAGCATAA
AACCAGTCAGCGAGCGGTCAGAAAAGATGCCGTTTTAGTCAATGAATGGATTATTTCGAGCGATA
GCAATTTCTTTGCTAATTTAACGGCGGCTGATACGCGCAAATATTTTGAAACAGCTAAAGCTTAC
TTTGCTGAAAAATTTGGTGAAGAAAACATTCGTTATGCGATTGTTCATCTTGATGAGAGTACGCC
CCATATGCATATGGGAATTGTTCCATTTGATGATGAGTATAAATTATCGGCTAAACGGGTGTTTA
ATCGTGCGGCTTTGC

8.11 pF880 sequence

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   1 gatcggattt gaaccgatgc cttcctattg atgtaatttt aatcattcca atcaactgtt
  61 tccgtgttgc cagattgtaa tgccttattg ttgtaactct gtaatttatc cgcttttatt
 121 tgttcttctg atgtccgatg attagcttgc atttcgaaca aaaacccaaa acaacccgga
 181 ataaatgata ataataccca ataacttcca gaaattgcga tacatgcaat tattgtaaat
 241 attgaaaata agattccttg ataatgattt cttattgcat gcagaaaagt tatatgctca
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 361 ttgattgtaa taaaataaaa agacccatat aacaacattg attctacttc ataaatcaat
 421 aaatctaaga atttcatacc gtttgtcaaa tgaaagacag tatcgaccaa actttgacta
 481 aagtaacaga ctaacgttgg taataacata aataattgtg ttactagtgg aatgtgcaac
 541 actggacaaa ttatactagt aataactaag ctaatatatt gaaaagcact tattggaact
 601 gatagggtaa aacaaaacaa gtcgattttt tgatagagac ttatggtctt tgacttgatg
 661 attctcgccg aatatttttt aaaaaattca aaatttgcag aacaaaactt actagatcta
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 781 ttaatgttcc agccttttag tgtagcttca atagaagtac aaaggtcttc tgccaccgca
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2761 aacggtacca gaatataaag ccatgcggga atctattaag caggggcagc aaaagttagc
2821 agcggctgaa aatgaaacta aacagcgtca agtgaaactt aaaacgtatc aagccactaa
2881 atttgatgtg aacagtgtta aaacgaagga atcccgtttc cataagcggt atgtgttggt
2941 tgaccgtttt gactctgata aacttaaaca gggcgcaagc ttaacggata cgtattttac
3001 tgaaacattg tcccaaaggt ctgatatgga tattcaaaaa gaccagttga tcaaagctga
3061 aagtaaggct atggaactag acattgagaa tcgtcgtctt caaaagttag ttgggacgct
3121 acaaggaatt gttcggagcg ttgaccgctt cttacagcgc aaattagggg ttggcttacc
3181 aagtaagtgg ctagaacgag ctggactaaa agaaccgtct aaaaaagccc ctcagaggcc
3241 acaggagcgt tcagagggac aacatgatga attagatggt ccaagtcttt gaatttggtc
3301 tatgacttta atttacccgc tgatgagcgt tggagctggt taatggccgt cagtcaacgg
3361 taaatcgaat taaagggact tactgcttta gcagttagtc ccttttgag gctttaagga
3421 gttgattgac ttactagacc aagacacttt tgcgcatgca aagaaaagca accctgcttt
3481 ttttgcctgc ctcacggcga gtgcggggtg agtttgagcg ggggctcccg ctcatttagg
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3541 cggtcaagct ggcatagctt ggactgggtt cggggcgtca gcgcccgagg gcttagaaac
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3661 aaactgggat aaatccttga cacaactggg cttaggcgta ttatgagttt ataaaatgaa
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4261 ttgaagagtg agttacggca gatgggacga gccattgcaa agatctttca gtataaaaaa
4321 gtggctaaaa atttgttggg ttatgtacgt tcaactgagg ttaccattaa tcacgaagca
4381 gatcagccga tgtatcacca ccatatgcat gttttgcttt ttatgaaatc gagttatttt
4441 acaggaactg ataattatat ttcacaagca gaatggaccg ggtattggca acgagcgatg
4501 aaattagctt atgtgccggt tgtgaatgtt gaagcggtta aaccgaatgt gaaacgccag
4561 aaaaattcct tgctggctag tgcccaggaa acggctaaat atcaggtaaa gtccaaagat
4621 attttaacta ataatcaaga acaagatcta caagtaattg atgatttgga acaagctttg
4681 gctggttccc ggcaaattag ctatgggggg ttgctgaaag aaattcgcaa gcagttgcaa
4741 ttggaagatg ttgaaaatgg tgatttgatt aatacggata gtgatgatca gcaggttgat
4801 caagcggtat gcgagattgt tgctaaatgg gactatcaac gcaaaaatta ttttatctga
4861 tgacttgttg cttatattat aagtgaaagg taggtagttt cgatgacttg ttttttggat
4921 tggttgaagg gaatacttag ttctactttt aatgtctctg tgaaaagtag aaagatgagt
4981 gataattctg gtacttttaa cggtaataaa gttaagaata tgggaaatgt tgatattaag
5041 ggtaacgaaa gagaggagaa aaagtagtat ggactatcaa aagagtcagt cttcaaataa
5101 ttttggtgtg ggtactggaa attcttttga aaagatgcat gattttattt tatagatatt
5161 gccgtgatct ttaaaatatt aacgtttgcg ggaatatcta tatctttatt ataattgtat
5221 tttgatgtta cttcaatttg agattagtgg caataatatg attttagtgc atgaaatgga
5281 ctgatttaac gttaagtggc atgctattat atttacattt acgttaaaat tggtattgct
5341 gatattttag taatttctcc tttttgtgtt tttacggtag aattagtttt taaattacat
5401 aagtttttg cgacgtgggc ttttttcacg tcgttattat gtattttgag ttatttgtga
5461 tttagtactg aagtttgttg cggtgttatt gaaaaaaaaa gacctactct ttacgagtaa
5521 gtctttaatt attgattgaa ttattgctca atgattca
```

8.12 Homology between pF8801 and pedA gene (ClustalW)

```
pF8801  GTGCGGGGTGAGTTTGAGCGGGGGCTCCCGCTCATTTAGGCGGTCAAGCT 3550
        --------------------------------------------------

pF8801  GGCATAGCTTGGACTGGGTTCGGGGCGTCAGCGCCCGAGGGCTTAGAAAC 3600
        --------------------------------------------------

pF8801  TGGTCGATTTTGACCAGTTTCTTCTTATCTTGATACTATTAGAAACAACG 3650
pedA    ----------------------------------CCGCAGAAGA-ATCA 14
                                           *      * * * *

pF8801  TCATTTTAAAAAACTGGGATAAATCCTTGACACAACTGGGCTTAGGCGTA 3700
pedA    TCAGGT-AATGTTTTGG-AAGAAT---TAAA-TAATGCAGCT-TG--GTA 55
        ***   * **     *** *  ***   * *   **    ***  *  ***

pF8801  TTATGAGTTTATAAAATGAATAAAG-AAAAAACCCACGTGAGAATTC-CT 3748
pedA    TGATGTGGTGGTAA-GAAGATCAAG-AAGAGTTCTA-GTGGTGACAT-TT 101
        * *** * *  ***     ** *** ** *   * * ***   *     *

pF8801  AGTTTGGCGACCCGGAACACGTGAGTTAATCTTGAATATTCGTATTTACT 3798
pedA    ACCTTAAC------AAGTGAATGTGATCATTTG-GCAACATG-------- 136
        *  **  *       *     ** * * ** *     *   *

pF8801  AGACATAGTTTAAAGCTTGAGTTAGTGAGCGTCAAGCCCTTAGTTTTAGT 3848
        --------------------------------------------------

pF8801  AAATACATAAAAGATTAGCTCTTCTCACATGGTTGGATGAGAGGAGCTTT 3898
        --------------------------------------------------
```