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The biological cost of mobile genetic elements

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ABSTRACT

The emergence and increase of antimicrobial resistance among bacterial populations has raised interest in the factor that contributes to this situation. Mobile genetic elements and in particular integrons have been found to play a role in the spread of resistance genes due to their ability to capture and integrate one or more gene cassettes by site-specific recombination. The genes can be integrated within the same attachment sites resulting in the formation of composite clusters of antibiotic resistance genes that facilitate multiple resistance phenotypes.

The aim of this study was to test the hypotheses that stated that the widespread dispersal of integrons in gram-negative bacteria is due to (1) low if any biological cost associated with harboring a class 1 integrons, (2) integron encoded cassettes are stably maintained in the absence of antimicrobial selection. The class 1 integron was obtained from the clinical strains *A.baumannii* Ab64 and Ab65 FFC and integrated into *A.baylyi* ADP1, an integron-free strain, by natural transformation. The fitness cost of three different strains was determined in pair wise competitions with the otherwise isogenic model organism ADP1. The strain Ab64.T1b that contained the newly acquired integron was found to have a fitness cost of 7% and stabile gene cassettes. The biological cost of the integrase was assessed with an integrase knock out strain Ab64.T1b *int::cat* and was observed to be 2% where as the fitness cost associated with harboring the gene cassettes *nptII and sacB* inserted in the selectively neutral locus was found to be zero. The stability of the integron encoded cassettes was tested in a 10 day experiment that corresponded to approximately 70 generation and it was found that the gene cassettes in the integron where 100% stable.

ABBREVIATIONS

59-be	59-base element
bla	beta – lactamase gene
BLAST	Basic local alignment search tool
CS	Conserved segment
ddNTP	Dideoxyribonucleotide tri phosphate
DNA	Deoxyribonucleic acid
HGT	Horizontal gene transfer
kb	kilo base pairs
K-res	Reference centre for detection of antimicrobial resistance
LB	Luria-Bertani medium
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
NCBI	National centre for biotechnology information
nptII /*aph* 3	kanamycin resistance gene
NTP	Nucleoside triphosphate
ORF	Open reading frame
P_2	Second integrase promoter
P_{ANT}	Promoter of integron
P_{INT}	Promoter of integrase gene
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
rpm	Revolutions per minute
UNN	University Hospital of North-Norway

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SECTION I: INTRODUCTION

1.1 Preface

In medical science, antibiotics are among the drugs that not only address the symptom but also have the ability to cure the disease by combating the source of infection. It is under the protective umbrella of antimicrobial agents that the greatest progress in the modern medicine has been encountered (Baquero and Blázquez 1997). The use of antibiotics has however come with increased resistance to these antimicrobial agents and it is increasingly being recognized that antibiotic consumption is among the major causes of the emergence of this resistance (Goossens et al. 2005). In nature, bacteria encounter ever changing and sometimes hostile environments. In order to survive, it has therefore been essential that bacteria develop highly sophisticated mechanisms to detect, evolve and adapt to these ever changing conditions. Some of these adaptations are mediated by complex processes both at the level of transcription and translation leading to alterations in gene expression patterns (Chowdhury et al. 1996). The development and use of antibiotics which are designed to inhibit bacterial growth and eliminate bacteria have in the same way evoked adaptations in bacterial populations hence the emergence of antibiotic resistant bacteria. Resistance may be classified as intrinsic or acquired. Intrinsic resistance in a bacterial population occurs when each member of an entire bacterial populations is resistant without any additional genetic alteration as is the case with beta-lactam resistance in *Mycoplasma* which is due the absence of peptidoglycan as a cell wall component (Normark and Normark 2002). The ability of bacteria to acquire traits that enable them to withstand antimicrobial agents has enabled bacteria to survive under antimicrobial selective pressure. The fact that the origin of majority of the compounds used to develop antibiotics are antibacterial compounds that are produced by microbes to protect them and their territories is believed to increase the ability of bacterial to develop mechanisms for survival in the presence of antimicrobial pressure (O'Brien 1997).

1.2 Antimicrobial agents

The era of antimicrobial agents began in the 1920's with Fleming's discovery of penicillin, a substance that had the ability to kill bacteria (Fleming 1929). Since then great numbers of antimicrobial agents have been discovered and developed. Antimicrobial agents can be broadly divided into two groups namely those that kill the bacterial cells (bactericides) or those that prevent the growth of bacteria (bacteriostatics) (Pankey and Sabath 2004). Another common method of classification is based on the mode of action and this divides them into antibiotics that interference with cell wall synthesis; inhibition of protein biosynthesis; interference with nucleic acid metabolism and inhibition of metabolic pathway (Tenover et al. 1997).

Interference with cell wall synthesis: can be achieved by interfering with the components required for the synthesis of the peptidoglycan layer by antibacterial drugs such as beta lactams. This group includes drugs such as the penicillins, cephalosporins, carbopenems, and monobactams, and glycopeptides, including vancomycin and teicoplanin (Tenover et al. 1997).

Inhibition of protein synthesis: can be achieved among others with macrolides, aminoglycosides or tetracyclines that bind to the 30S subunit of the ribosome. Chloramphenicol provides the same effect by binding to the 50S subunit (Tenover et al. 1997).

Interference with nucleic acid metabolism: Lethal double-strand DNA breaks during DNA replication can be effected with the use of fluoroquinolones (Tenover et al. 1997).

Inhibition of metabolic pathways: Sulfonamides and trimethoprim prevent DNA synthesis by interfering with two steps in the bacterial pathway for folic acid synthesis (Tenover et al. 1997).

The main mechanisms of acquired resistance can be categorized as: (a) Alteration of a compound or a pathway; (b) over production of the drug target; (c) increased efflux of antibiotic from the cytoplasm; (d) reduced antibiotic uptake into the bacterium and (e) enzymatic modification or destruction of antibiotic (Barker 1999).

1.3 Resistance development

Antibiotic resistance in bacteria is developed either by de novo genetic changes (mutations) within the organism or by the acquisition of genetic material by horizontal gene transfer (HGT) from external sources.

Mutations are defined as any changes that occur in the DNA sequence of the genome of an organism and result in differences when compared to the wild type strain (Maloy et al. 1994). Although mutations are often the main cause of de novo changes and occur naturally (Low et al. 1999), they are very rare events and are only known to occur at rates of 10^{-9} to 10^{-8} per generation (Freifelder 1987). Mutations in a single gene may result in resistance without altering the pathogenicity or viability of a bacterial strain. However further mutational events may alter the existing mechanisms of resistance making them either more active or providing a broader spectrum of activity (Gold and Moellering 1996). It is likely that some of the resistance genes that are currently spread among bacterial populations resulted from mutations that were selected for by use of antibiotic or naturally occurring antimicrobial agents (Amábile-Cuevas and Davies 2003). However as mutations are rare, it is evident that other mechanisms are involved in the development of resistance.

1.4 Horizontal Gene Transfer and mobile genetic elements

Horizontal gene transfer is defined as a process by which an organism acquires genetic material from another organism. It is a natural phenomenon that was first demonstrated to occur in streptococcus by Griffith in 1928 (Syvanen 1994). At present, three mechanisms of HGT have been described namely, transformation, transduction and conjugation. HGT plays an important role in the acquisition of new traits in a bacterial population and it is known to be associated with the emergence of multi resistance to antibiotics (Ochman et al. 2000). The development of resistance by the acquisition of external genetic material or HGT is of greater concern as it may occur at higher frequencies of 10^{-5} to 10^{-4} per generation (Freifelder 1987). High rates of HGT may undermine the reversal of resistance as strains within the same population that were initially susceptible are directly provided with resistance determinants from resistant strains and conjugative elements harboring antimicrobial resistance determinants and may thus avoid host selection by rapid transfer to other hosts that many provide more habitable condition (Johnsen et al. 2009).

1.4.1 Transformation

Transformation is a process by which a bacterial cell actively takes up foreign DNA from its surroundings, integrates and expresses the genes located on it. This process does not require a living donor cell as the release of DNA during death and cell lysis suffices to provide free DNA (Lorenz and Wackernagel 1994). It is however restricted to naturally transformable (competent) prokaryotes. The process of transformation requires the development of cell competence; DNA binding; DNA uptake and stable genomic integration, and expression of the acquired genes (Baur et al. 1996). Competence in a bacterial cell is a physiological state that allows the active uptake of DNA (Baur et al. 1996). In some bacterial species such as *Neisseria gonorrhoeae* competence is constitutively expressed (Biswas et al. 1977) whilst other transformable species such as *Bacillus subtilis* competence is only achieved at specific stage of growth such as the late log phase (Maloy et al. 1994). Transformation in bacteria is largely limited to situations where the incoming DNA can reconstitute to form a self-replicating entity (e.g. plasmid), or where there is sufficient sequence homology with the recipient chromosome to allow insertion by recombination. Since many of these bacterial species are promiscuous in their uptake of free DNA, gene transfer by transformation between even distantly related bacteria is possible (Cummins et al. 2000).

1.4.2 Transduction

Transduction is a bacteriophage particle mediated process in which genetic material is transferred from one bacterial cell to another. The life cycle of a bacteriophage includes a step of surface attachment to the bacterial cell and injecting its DNA into the cytoplasm. When this DNA is expressed, an infection cycle can be started which leads to production of new phage particles which are released from the infected bacterium and go on to infect other cells. In rare cases a new particle may carry bacterial instead of viral DNA and deliver it to a second bacterium can that incorporate it into its genome. Phage particles are capable of transferring whole plasmids and pieces of chromosomes between hosts (Read 2000).Transduction can occur in a wide range of bacteria however as the success of the process is based on the ability of the bacteriophage to infect the recipient, the transfer of DNA is usually restricted to related species (Kokjohn 1989).

1.4.3 Conjugation

Conjugation is a process by which conjugative elements directly transfer DNA from donor to recipient cells with the aid of protein complexes which connect the donor to the recipient cells. In this process, cell to cell contact is required as the DNA is transferred directly from donor to recipient (Mascaretti 2003). Conjugation provides a means of transferring genetic material not just between species that are closely related but also distant species hence it is assumed to play a major role in the acquisition and spread of resistance among bacterial populations. This spread of resistance is mediated by two types of broad-host-range elements, namely conjugal broad-host-range plasmids and conjugative transposons (Salyers and Amabile-Cuevas 1997) Conjugal plasmids are extra chromosomal elements that carry the necessary DNA to mediate their own transfer during conjugation. The integration of plasmids into the chromosome is very rare as the acquired trait must confer an advantage large enough to overcome inactivation by mutation and elimination by segregation as is the case with antibiotic resistance genes (Ochman et al. 2000). Conjugative transposons were first discovered in gram-positive cocci and *Bacteroides* spp. but have now been found in a wide range of hosts (Salyers and Amabile-Cuevas 1997). They are generally described as DNA segments that range in size from 18 to over 150 kbp and have the ability to form a non-replicating circular intermediate that is transferred to the recipient by conjugation. This non-replicating intermediate is integrated into the genome of the recipient by an integrase which is encoded by the conjugative transposon (Salyers and Amabile-Cuevas 1997).

1.5 Mobile genetic elements

The movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility) is mediated by enzymes and proteins that are encoded by genes located on segments of DNA that are called mobile genetic elements (MGEs) (Frost et al. 2005). MGEs are involved in the spread of adaptive and symbiotic traits involved in host survival (Mark Osborn and Böltner 2002). The genes encoding transposases and site-specific recombinases found in MGEs facilitate their movement within the genome which can lead to genomic rearrangements (Frost et al. 2005). Currently it is apparent that MGEs such as plasmids, transposons and bacteriophages provide key vehicles for gene transfer between bacteria (Lanka and Wilkins 1995) and hence contribute directly to changes in bacterial population dynamics.

1.5.1 Transposons

Transposons are mobile genetic elements which are sometimes referred to as 'jumping genes' due to their ability have the ability to be move from one location in the genome to another or across genomes (Snustad et al. 1997). They were first revealed in a genetic experiment based on maize conducted by McClintock (Georgiev 1984) but have since been found to constitute large percentages of the animal, plants and bacterial genomes (Kazazian Jr 2004). The transposon encode the genes required for self translocation (Carattoli 2001). Currently three types of transposable elements have been identified, namely; insertion sequence (IS) elements, composite transposons, and non-composite transposons. Several transposons are known to contribute to bacterial genome evolution by excision and insertion of DNA from a donor site to other non homologous target sites and they have the ability to promote the transfer of antibiotic resistance genes between bacterial genomes (Ochman et al. 2000).

1.5.2 Integrons

Integrons are naturally occurring gene expression elements that where discovered by Stokes and Hall (Stokes and Hall 1989) and have been defined as specialized genetic structures that are responsible for the acquisition of resistance genes. Integrons are distinct from all other genetic elements in that they are able to utilize site-specific recombination to acquire and integrate circular gene cassettes (see section 1.5.3) (Collis and Hall 1992a).

Integrons contain two specific elements that is, the integrase gene (*intI*) and the *attI* site which are both located at the 5' conserved segment (CS) of the integron. These two elements enable the integron to insert and excise gene cassettes by *intI* catalyzed site specific recombination (Collis et al. 1998). The *intI* gene encodes for an integrase which is a member of the tyrosine site specific recombinases and the *attI* site is the incorporation site or recombination site for the incoming genes (Recchia and Hall 1995). The *intI* mediates recombination between the primary recombination site (*attI*) and the secondary target *attC* which is normally found associated to single open reading frames or gene cassettes. The other key feature of the integron is the strong promoter P_{ant} that is located upstream of the integration site, (Figure 1) which illustrates a simplified structure of class 1 integrons (Martinez-Freijo et al. 1998). The cassette genes are expressed from this promoter and their expression is based on the correct orientation from the promoter. All the cassettes in a an integron are expressed from a common promoter with the ones closest to the promoter having higher expression levels (Martinez-

Freijo et al. 1998). The insertion of three nucleotides between the relatively close -35 and -10 regions has resulted in a second promoter P_2 which has been found in conjunction with the weakest variants of P_{ant} (Hall and Collis 1995).

1.5.3 Gene cassettes

Gene cassettes are the smallest known mobile elements and have generally been found to consist of a coding region (open reading frame) and the "59 base pair elements" (59-be) or *attC* site (Collis and Hall 1992a). The *attC* which is located at the 3' end of the gene is a recombination site that is recognized by the integron site specific integrase *IntI* (Collis and Hall 1995) and is thus responsible for the mobility for the cassette. Gene cassettes are found in two forms namely as part of an integron or as separate small circular molecules. In their circular form, gene cassettes are unable to replicate or express the gene they are carrying as they do not contain a promoter (Stokes et al. 1997). Once integrated at the *attI* site of the integron, the genes in the cassette may be expressed by the promoter that is located upstream from the cassette provided that the cassette is in the correct orientation (Recchia and Hall 1997). The 59- be of each gene cassette differ in length and sequence and are therefore unique for each specific cassette (Collis et al. 1998). Currently, all known 59-bes have common inverted repeats structures and comparison of the 59-be sequences has shown that the most conserved segments lie with two regions of over 20 bp at the ends related to consensus sequence. These two regions which are imperfect inverted regions of each other are separated by sequence that varies in length and sequence (Stokes et al. 1997). The most conserved region of the 59-be is the seven base pair core site GTTAGGC which is located on the right hand end of the element and the inverse core site GCCTAAC or RYYYAAC located at the left hand end of the element (Collis and Hall 1992a). Gene cassettes were first identified in relation to antibiotic resistance and as of 2009 at least 130 gene cassettes had been discovered and characterized (Partridge et al. 2009) .

Integrons have been found association with to mobile genetic elements that confer multiple drug resistance in pathogenic bacteria isolated from humans, animals and plants (Ilyina 2006). Their ability to capture one or more gene cassette within the same attachment sites has led to the formation of composite clusters of antibiotic resistance genes which facilitate multiple resistance phenotypes (Carattoli 2001). The range and number of antibiotics that an integron carrying strain may be resistant to varies as it is dependant on the number and type of resistance genes present in the integron (Ilyina 2006). Integrons do not contain the machinery

needed to transfer themselves from one part of the genome to another. It is therefore not surprising that they are commonly found associated with insertion sequences and or composite transposons that are present in transposons and plasmids. This association provides a means of movement and hence the antibiotic resistant genes that are often found on the gene cassettes are transferred inter- or intra species (Boucher et al. 2007).

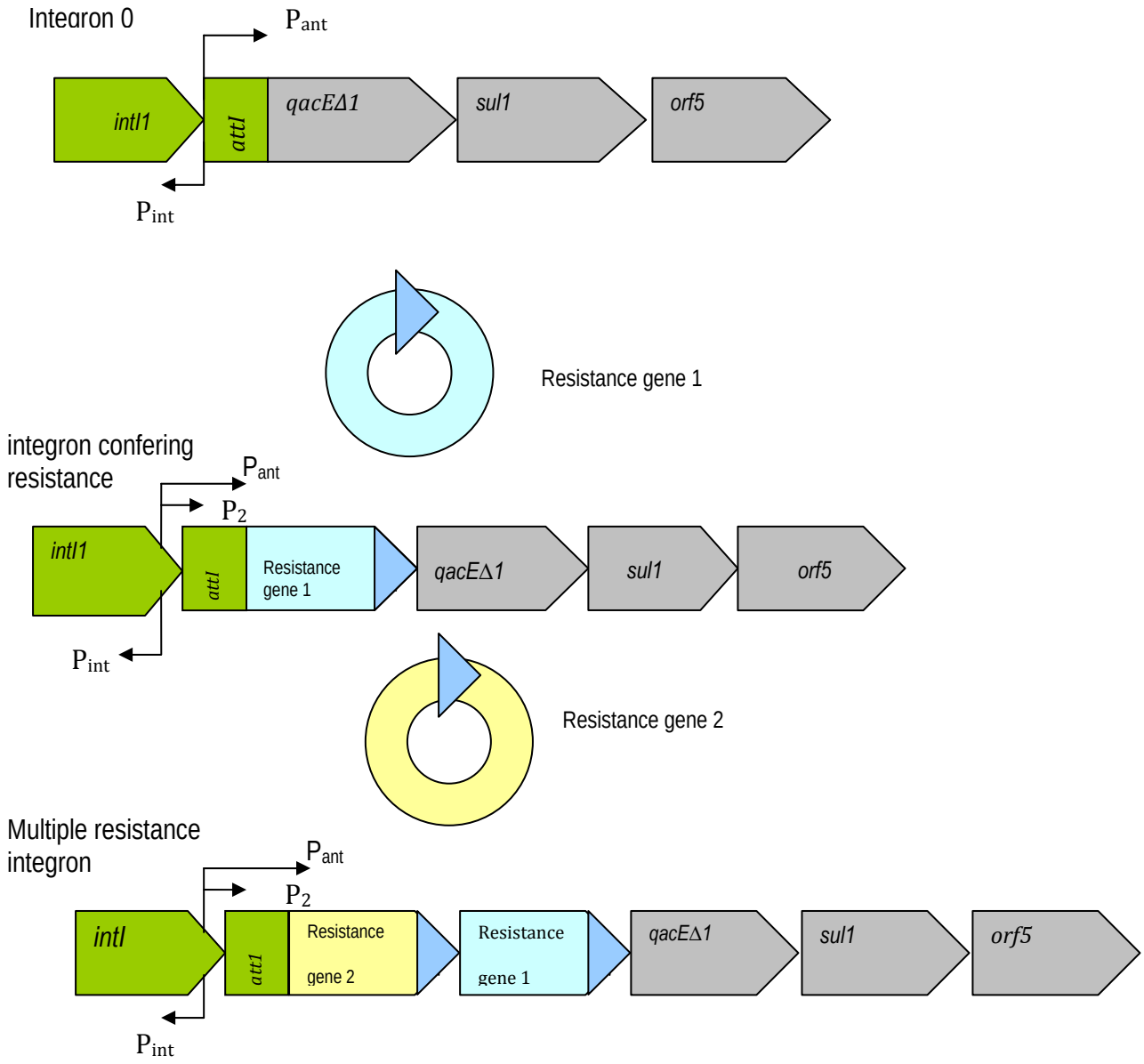


Figure1: A simplified schematic representation of a gene cassette acquisition model in class 1 integrons. The integrase gene *intI*1 and a*ttI* 1 constitute the 5' conserved segment (5`CS) where as the 3`conserved segments (3'C) is composed of the *qacE*Δ1 which encodes for quaternary ammonium compounds; *sul1* which encodes for, sulphonamide resistance and the *orf5*, a gene of unknown function. The promoter P_{ant} is transcribes the gene cassettes; P_{int} is responsible for the transcription of the integrase and P_2 is a second promoter which has been identified in integrons with weaker P_{ant} promoters. The gene cassettes 1 and 2 represent gene cassettes that confer resistance to difference antimicrobial agents that may be captured and integrated by the integron. Structure outline adopted form Carattoli, Fluit and Schmitz (Carattoli 2001, Fluit and Schmitz 1999).

Integrons play a major role in the evolution of bacteria as they are able to capture and integrate gene cassettes which contain functional genes and hence mobilize them among many individuals in a population in a way that they can be expressed without disrupting the expression of other genes in the cell (Michael et al. 2004). Once integrated and expressed in the integron, the resistance genes may be spread across phylogenetic boundaries through their association with the integrons. The association of integrons with mobile genetic elements such as plasmids and transposons has resulted in a wide distribution of the resistance genes that are carried on the integron among gram negative and some gram positive bacteria (Boucher et al. 2007). The role that integrons play in the evolution of bacteria is attributed to their ability to change and adapt to different functional niches in various host organisms. The degree of mobility, phylogenetic distribution, amount of coding content in cassettes, the diversity and size of the gene cassette and the rate of gene cassette loss and acquisition are some of the variables that enable integrons to undergo the required changes (Boucher et al. 2007).

Integrase

Integrases are members of the tyrosine recombinase family of site-specific recombinases (Esposito and Scocca 1997) and are responsible for many important biological processes including regulation of gene expression and DNA replication, plasmid copy number maintenance, conjugative transposition, catenated circle resolution, daughter chromosome segregation, and prokaryotic telomere processing (Mumm et al. 2006). The site specific recombinases which could lead to diverse genetic rearrangements including integration, inversion or excision of DNA molecules achieve their function by interacting with short sequences in the DNA, bring two sites together in a synapse and then catalyzing strand exchange so that the DNA is cleaved and relegated to opposite partners (Nash 1996).

Classification of Integrons

Integrons have broadly been divided into two groups namely resistance integron (mobile integrons) which carry genes that code for antibiotic resistance and super integrons carry genes with a variety of function (Boucher et al. 2007). Resistance or mobile integrons can be located on a chromosome or on a plasmid whilst others are located on the chromosome (Fluit and Schmitz 2004). The structure and the function of an integron are similar regardless of

whether it is located on a chromosome or on a plasmid (Gillings et al. 2005). Mobile or resistance integrons have further been divided into five classes based on the differences in the sequence of the integrase gene *intI* (Rowe-Magnus and Mazel 2002).

Class 1 integrons are the most studied and as the name suggests they were the first group to have been discovered. They are typically located on plasmids or transposons which enable them to move and their importance in the disseminating antibiotic resistance genes in both gram negative and gram positive bacteria is well documented (Nemergut et al. 2008). The structure of class 1 integrons is well described and is comprised of two conserved segments, the 5' conserved segment (5'CS) and 3' conserved segments (3'CS). These are separated by a variable region into which gene cassettes are inserted. This region in which genes can be inserted has been found to contain distinct antibiotic resistance genes and some unidentified open reading frames and the number of cassettes on an integron seem not to be limited as several inserted genes have been found in nature (Recchia and Hall 1995). The 5' CS contains the *int1* gene which encodes the class 1 integrase, the gene that is responsible for site specific insertion and excision of gene cassettes (Tosini et al. 1998). The 5'CS also contains the cassette insertion site *attI* and the promoter which facilitates the expression of the genes contained in the cassettes (Bennett PM 2008). The 3'CS has been found to contain the *sul1* and *qacEΔ1* genes which encode for resistance to sulfonamides and quaternary ammonium compounds respectively (Tosini et al. 1998). In addition to these, the 3'CS has been found to contain *orf5* which encodes for a protein of unknown function (Carattoli 2001). As gene cassettes do not contain promoters, the promoter located on the 5' SC is a necessity in the expression of these genes in addition to the orientation of the cassettes. Acquisition of additional cassettes occurs by integrase mediated recombination between *attC*, in the incoming cassette and the *attI* site closest to the integrase as described in section I: 1.5.2, 1.5.3 as well as Figure 1. Cassettes can be inserted one after the other at the insertion site attI to give a multi resistance integron (Bennett PM 2008).

The class 2 integrons that are currently known are embedded in the Tn*7* family of transposon (Fluit and Schmitz 2004) and have a putative defective integrase gene (*int2*). The *int2* is located in the distal portion of Tn*7* and its product is 40% identical to that of *int*1 (Tosini et al. 1998). The Tn*7* usually contains three integrated gene cassettes namely *dhfrI-sat-aada1* which are adjacent to *int2* and *attI* site which is located between the first inserted resistance genes in class 1 integrons. Class 2 integrons contain genes that promote the Tn*7* transposition and they do not contain the *sul1* gene (Carattoli 2001). The presence of a stop codon at amino

acid 179 in the class 2 integrase (*int2*) is assumed to be the cause of the decreased diversity that has been exhibited by class 2 integrons. The stop codon is suspected of producing a shorter and probably inactive polypeptide that is unable to catalyze the integrase meditated recombination reaction observed in other classes of integrons (Barlow and Gobius 2006).

In class 3 integrons a putitative integrase (*int3*) that is located at the 5'of the bla_{IMP} cassette has been identified. The sequence of this gene has been found to be up to 61% identical to that of the class 1 *int1* gene (Tosini et al. 1998). The gene cassette in this integron contains part of the *aacA4* gene that was previously identified as a gene cassette in class 1 and the bla_{IMP} gene cassette that confers resistance to broad spectrum beta lactams including carbapenems (Carattoli 2001). A large transferable plasmid in a *Serratia marcescens* strain is the only known example of a class 3 integron platform (Rowe-Magnus and Mazel 2001)

Class 4 integrons have been identified in *Vibrio cholerae* and sequence analysis of this integron suggests that it may have been acquired through integrase-mediated recombination between the *attC* site of the last gene cassette a secondary site in the constin (which is a conjugative self-transmissible integrating element) of which the integron is a components of (Rowe-Magnus and Mazel 2001).

A fifth class of integrons has been identified on the plasmid pRVS1 of a *V. salmonicida* and it carries a single antibiotic resistance cassette followed by several other cassettes whose function seem not to be related to antibiotic resistance (Rowe-Magnus and Mazel 2001). These integrons are all known to be physically linked to mobile elements such as insertion sequence transposons and conjugative transposons which mediate their spread among species (Mazel 2006).

Super integrons have been identified in *Vibrionaceae*, *Shewanella*, *Xanthomonads*, *Pseudomonads,* and *Nitrosomonads* (Rowe-Magnus and Mazel 2001). They are mainly distinguished by the large number of gene cassettes present, many with unknown functions as well as their stable association with the chromosome. The gene cassettes in these integrons exist as autonomous non-replicating structures that contain one open reading frame and one *Vibrio cholerae* repeat element sequence capable of repeated integration into both its own integron and other integrons with the aid of the integrase (Ilyina 2006).

2.0 The reversal of antimicrobial resistance

The resistance to antimicrobial drugs in bacterial populations represents one of the most serious and discussed challenges encountered in antibiotic therapy. The amount of antibiotic use, the fitness costs of resistance to the bacteria coupled with the ability of bacteria to compensate for the cost encountered by harboring resistance traits are among the major factors that determine the frequency of resistance in a bacterial population (Andersson and Levin 1999). Of these three parameters, the biological cost that is exerted on the bacteria by the acquired resistance, is the focus of this study.

2.1 Removal of drug selection

The association between the use of antimicrobial agents and the emergence of resistance is clear (Goossens et al. 2005), however at present there is very limited information concerning the effects of removing antimicrobial selection on the occurrence of antimicrobial resistance (Aarestrup et al. 2001). Johnsen and others used the figure below (figure 2) to illustrate the possible outcome of removal of drug selection (Johnsen et al. 2009). From the figure it is seen that in the presence of selection, the frequency of resistance is maintained until the selective pressure is removed. If maintaining the genes that confer resistance exerts a biological cost on the host bacteria, the frequency of resistance would be expected to decline to the level where resistance isre-acquired by mutation or HGT. The compensatory evolution would enable the bacteria to maintain low but constant frequencies of mutation until the selective pressure is re-introduced. At this point the frequency of resistance would be expected to rapidly increase.

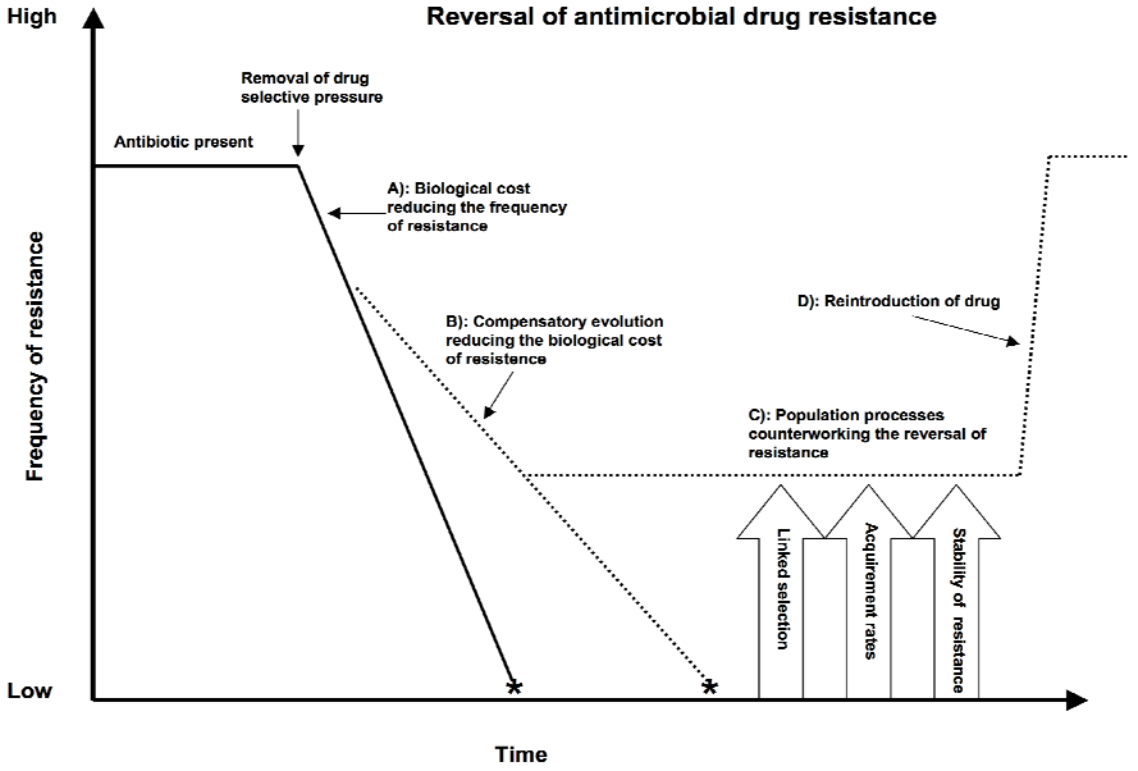


Figure 2: A representation of the processes involved in the reversal of antimicrobial drug resistance in bacterial populations. The figure shows that in the presence of selective pressure resistance frequencies are maintained. Upon removal of selective pressure the resistance frequency declines and if maintaining the resistance genes infers a cost on the bacteria then resistance is expected to be totally lost from the population (A). This is provided that the population does not experience compensatory evolution, in which case (B) would occur. (Adopted and modified from Johnsen et al 2009).

2.2 Fitness cost of resistance

Fitness in bacteria is a concept that implies the existence of heritable variations among individual members of a species and in infectious pathogens it is a composite measure of the ability of an organism to survive, reproduce and be transmitted (Cohen et al. 2003). The characteristics of an organisms` growth when in its host, its ability to withstand environmental stress which may be present within the host or in the hosts environment and the capacity of the organism to disseminate and establish in a new trait in a host may all be used as measures to determine fitness (Cohen et al. 2003). Fitness may be defined in terms of absolute fitness or relative fitness. Absolute fitness describes how sensitive or resistant bacteria grow, are transmitted between hosts, and cleared from infected hosts whilst relative fitness refers to the relative rates at which resistant and sensitive bacteria grow and die (compete) in hosts and environments (Bennett AF and Lenski 1993).

2.3 Compensatory mutations

Mutations that lead to resistant phenotypes normally occur in genes that are essential to the cell thereby affecting the natural function of the gene. This infers a biological cost on the cell (Andersson and Levin 1999). It is well established, that instead of reversion mutations that restore susceptibility, bacteria may reduce the biological cost of resistance through compensatory mutations (Normark and Normark 2002). These mutations occur normally at higher rates than simple reversions due to the fact that more compensatory mutation loci exist than susceptibility reversions, reviewed in (Johnsen et al. 2009).

3.0 The Model organism: *Acinetobacter baylyi* strain ADP1

The genus *Acinetobacter* is now defined as including gram negative coccobacilli, that are strictly aerobic, nonmotile, catalase positive, and oxidase negative (Bergogne-Berezin and Towner 1996). They have been found in a wide range of environments including aquatic bodies, sewers and in living organisms (Barbe et al. 2004). Members of the *Acinetobacter* genus are known to be nutritionally versatile and the range of substrates that can be used as sole carbon and energy sources is wide. The relative ease and simple culture requirements coupled with their involvement in the biodegradation of a pollutants and their role as agents of nosocomial infections has drawn a great amount attention to this genus (Barbe et al. 2004).

A. baylyi is a naturally transformable, gram-negative and prototrophic bacterium with simple culture requirements and a compact genome of 3.6 Mb (Barbe et al. 2004). BD413 or ADP1 (as the strain is called in Europe and USA respectively) is a mini-capsulated mutant that was obtained by UV irradiation of the original strain BD4 (Barbe et al. 2004) which was originally isolated from a soil enrichment whose main carbon source was 2.3-butanediol (Juni and Janik 1969). It is highly transformable, that is it readily takes up foreign DNA without bias towards the source (Palmen et al. 1993) or discrimination between homologous and heterologous DNA (Nielsen et al. 1997). ADP1 also has simple culture requirements and is known to grow slowly at room temperature but optimally at temperatures ranging between 30°C and 37°C (Metzgar et al. 2004).

ADP1 was selected as the model organism in this study as it is fully sequenced and has simple culture requirements. The strain is also closely related to the more troublesome *A. baumannii*

which is an opportunistic human pathogen that has been known to cause serious infections in humans, especially in immuno-compromised patients (Tomaras et al. 2008). The number of multi-drug resistance isolates of *A. baumannii* are on the increase and currently isolates that are resistant to aminoglycosides, fluoroquinolones, and carbopenems have been reported (Garnacho-Montero et al. 2003). The growing number of community and nosocomial infections caused by *A. baumannii* coupled with the emergence of multi resistant strains raises the need to understand this microorganism better. Molecular studies of this strain has however been hampered by its multiple resistant phenotype (Ramirez et al. 2010) and the fact that it is not as easily transformable in comparison with *A. baylyi* (Chen et al. 2008). The construction of *A. baylyi* that contains an integron (that was originally in *A. baumannii*) and confers resistance to antimicrobial agents therefore provides a window to the world of epidemic outbreaks and endemic situations involving multi drug resistant *Acinetobacter.*

HYPOTHESIS

The widespread dispersal of integrons in Gram-negatives is due to:

1. **Low if any biological costs of harboring a class 1 integron**

2. **Integron encoded cassettes are stably maintained in the absence of antimicrobial selection.**

MAIN / PRINCIPLE OBJECTIVES

Determine the fitness cost of a newly acquired class 1 integron and investigate the gene-cassette dynamics of integrons of clinical origin as well as in a naive host.

SPECIFIC OBJECTIVES

1) Clone a class 1 integron from *Acinetobacter baumannii* (Da Silva G. J. et al. 2002b) into *Acinetobacter baylyi* and determine the biological cost of the integron and the stability of the resistance cassettes in both the presence and absence of antibiotic selection.

2) To determine the phenotypic and genotypic stability of the gene cassettes in class 1 integron in its native hosts (the clinical isolates *Pseudomona*s *aeruginosa* and *Acinetobacter baumannii*) and in the naive host *Acinetobacter baylyi*.

3) Experimentally recruit gene cassettes into a newly acquired integron in *Acinetobacter baylyi.*

SECTION II: MATERIALS AND METHODS

1.1 Bacterial strains

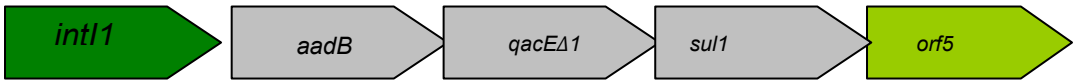
The stability and fitness of the class 1 integrons in this study was investigated with two *A. baumannii* strains Ab 65FFC (Da Silva G et al. 2002a) and 064Ab (provided by G. DaSilva, unpublished) and two clinical isolates of *P. aeruginosa* (provided by Ørjan Samuelsen, UNN, Tromsø). The *A. baumannii* strain 064Ab harbors an integron that contains a gene cassette (*aadB*) that confers resistance to aminoglycosides (gentamicin, kanamycin and tobramycin) while strain 65FFC has an integron that carries the bla_{imp-5} gene cassette that confers resistance to carbopenems and broad spectrum cephalosporins ceftazidime, ceftriaxone, cefepime and cefpirome (Da Silva G et al. 2002a).The Integron in the *P. aeruginosa* strain K34-74 has gene cassettes *aacA4*, bla_{vim-4} and bla_{oxa-35} that confers resistance to aminoglycosides and beta-lactams. The integron in strain A3-54 carries the bla_{imp-2} and *aadB* genes which confer resistance to aminoglycosides and carbopenems (Samuelsen et al. 2010). Figure 3 provides a schematic representation of the structures of the integrons in the strains used in this study.

1.2 Plasmids

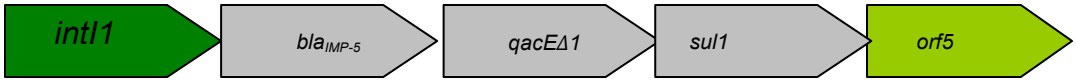
The construction of *A. baylyi* strains that contains a class 1integron required the use of a variety of plasmids. The plasmids used in this study are listed in table 1.

Table 1: A table containing the plasmids used in this study. The names, genes contained in the plasmid and the source of the plasmids are listed in the table.

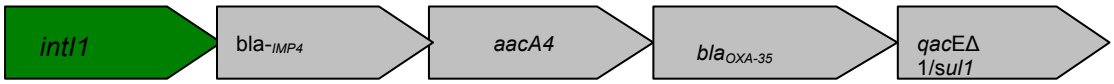
Name of Plasmid	**Relevant genotype and phenotypes**	**Source / reference**
pGT41	Derivative of pPCR-Script Cam containing *bla sacB nptII*, cat (Cm^R)	(Kickstein et al. 2007)
pTM1	pGT41:: down flank (*nptII*) Cm^R, Km^R	This study
pTM2	pGT41::down flank and up flank; (*bla, nptII*) Cm^R, Km^R, Ap^R	This study
pTM3	pGT41::Up and down flanks; Δ*sacB, nptII*	This study
pTM4	pGT41::Up and down flanks; Δ*sacB, nptII 'bla*(inactive) Cm^R	This study
pCYC177- int-cat	Cm^R, Ap^R, Δ*intI1::cat*	K. Harms, unpublished)



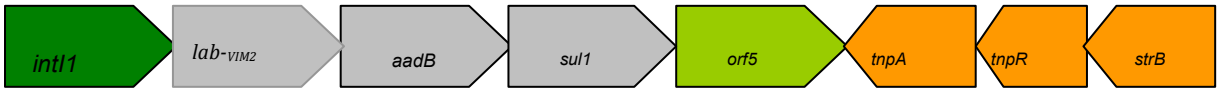
(i) A. baumannii 064 : A schematic representation of the class 1 integron found in *A. baumannii* 064 that carries the *aadB* cassettes that confers resistance to aminoglycosides (resistance to gentamicin, tobramycin, kanamycin but not amikacin). The figure illustrates the structure of the integron with *intI*1: integrase gene; *qacE*Δ1: quaternary ammonium compounds; *sul1*: sulphonamide resistance gene and *orf5* a gene cassette encoding for a protein of unknown function.



(ii) *A. baumannii* 65FFC: A schematic representation of the class 1 integron found in *A. baumannii* 65FFC containing class 1 integron that confers resistance to ampicillin; The structure of the integron with is shown in the figure with *intI*1: integrase gene; *qacE*Δ1: quaternary ammonium compounds; *suI*1: sulphonamide resistance gene and *orf*5 a gene cassette encoding for a protein of unknown function.



(iii) *P. aeruginosa* K34-73: A schematic representation of the class 1 integron found in *P. aeruginosa* containing gene cassettes that confers resistance to aminoglycosides (resistance to amikacin and tobramycin but not gentamicin) and carbopenems; The structure of the integron which is shown in the figure with *intI*1: integrase gene; *qacE*Δ1: quaternary ammonium compounds; *suI*1: sulphonamide resistance gene and *orf*5 a gene cassette encoding for a protein of unknown function.



(iv) *P. aeruginosa* A3-54: A schematic representation of the class 1 integron in *P. aeruginosa* A3-54 with an integron with multiple gene cassettes. Bla_{vim2} confers resistance to metallo beta lactams; *aadB* cassettes that confers resistance to aminoglycosides (resistance to gentamicin, tobramycin, kanamycin but not amikacin); *intI*1: integrase gene; *qacE*Δ1: quaternary ammonium compounds; *suI*1: sulphonamide resistance gene and *orf*5 a gene cassette encoding for a protein of unknown function.

Figure 3: Schematic presentation of the class 1 integrons that are present in the test strains used in this study.

Table 2: The bacterial strains that were used during this study. The table indicates the strain name, the application of the strain to this study and the source of the strain.

Name of strain	**Resistance gene present and/or genotype**	**Application in this study**	**Source**
A. baumannii 064	*aadB*	Transformation, Cassette stability	Da Silva unpublished
A. baumannii 65FFC	*bla-$_{IMP-5}$*	Transformation, Cassette stability,	(Da Silva G et al. 2002a)
P. aeruginosa K34-74	*aacA4, bla-$_{VIM-4}$, bla-$_{OXA-35}$*	Transformation, cassette stability and recruitment	(Samuelsen et al. 2009)
P. aeruginosa A3-54	*aadB, bla-$_{IMP-2}$*	Cassette stability	(Samuelsen et al. 2009)
Ab64.T1b	*aadB*	Cassette stability	This study
Ab.T1b.Da		Cassette recruitment	This study
Ab64.T1b*int::cat*	*cat, aadB*	Integrase study	This study
Escherichia coli SF8 *rec*A	Genotype: K-12; *recB21 recC22 sbcB thr-1 leuB6 thi-1 lacYl lop-l, tonAl supE44* rK- mK-; *recA56*	Plasmid isolation, electroporation assays	(Romanowski et al. 1993)
Escherichia coli EC100	Genotype: *F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (Str^{R}) nupG.*	Plasmid isolation, electroporation assays	Epicentre Biotechnologies, Madison, Wisconsin

1.3 Growth Media

1.3.1 Luria Broth Media (LB Media)

The LB media was prepared by dissolving 25 g of Luria broth Base from Invitrogen, Germany in 1000 ml of distilled water. The solution was autoclaved and stored at room temperature.

1.3.2 Luria Broth Agar (LB plates)

The Luria broth agar or LB plates were prepared by dissolving 25 g Luria broth base (Invitrogen, Germany) and 15 g of agar (Merck, Germany) in 1000 ml of distilled water. The solution was sterilized by autoclaving and after cooling to 55°C prior to the addition of the appropriate antibiotics in the required antibiotic concentration. The selective plates used in this study included kanamycin 50 µg/ml , ampicillin 100 µg/ml , chloramphenicol 25 µg/ml and sucrose (50 g/L) unless indicated otherwise.

1.4 Freeze Stock Solutions

Strains were streaked onto LB plates containing the appropriate antibiotic the plates and incubated at 37°C for 16 hours. Freeze stock solutions of all the strains were prepared by collecting 5-6 single colonies from LB plates and suspending the colonies in 5ml LB media prior to overnight incubation at 37°C with aeration. A volume of 900 µl of the overnight culture was mixed with 900 µl LB media containing glycerol (final concentration of 20%) and stored at -80°C.

1.5 Model organism

A. baylyi strain ADP1 (Barbe et al. 2004) was used as the model organism in this study. This strain is a naturally transformable gram-negative bacterium with simple culture requirements. It is prototroph with a compact genome of 3.6 Mb and is highly transformable, taking up foreign DNA during its growth without bias towards the source (Palmen et al. 1993). The construction of an integron carrying *A. baylyi* required the cloning of integrons from *P. aeruginosa* and *A. baumannii* by employing the plasmid pGT41 into a selectively neutral site

(P. Johnsen, personal communication) which was formally known as lipA-lipB locus (Kok et al. 1995) of *A. baylyi* . The former *lipB* gene (now *lifO* and annotated as ACAID3308) encodes for the chaperon (Barbe et al. 2004) and is from here on referred to as the up flank. The ACAID3309 (formerly annotated *lipA*) encodes a putative lipase (Barbe et al. 2004) and is from here on referred to as the down flank.

2.0 DNA Isolation

The isolation of genomic DNA from the bacterial samples was conducted by two methods, namely cell lysis and column purification. The column purified DNA was obtained using the Qiagen genomic isolation kits (QIAGEN, Hilden, Germany) where as the cell lysis method involved disrupting the bacterial cells at high temperature and collecting the cells contents. The column purified DNA was used for down stream applications such as transformation, PCR and subsequent sequencing whilst the DNA isolated by cell lysis was used mainly for transformant screening.

2.1 Cell Lysis

The chromosomal DNA was isolated from single colonies grown on LB plates (supplemented with necessary antibiotics for selection) after streaking the freeze stock and incubation at 37°C. The single colonies were suspended in 50 µl of PCR water and boiled in a PCR PTC-200 thermal cycler (BIO-RAD, Norway) machine for 10 minutes at 100°C. The suspension was then centrifuged at 13000 rpm for 5 minutes prior to transferring the supernatant that contains the cell material into a new tube. The supernatant was quantified and stored at -20°C until needed for downstream applications.

2.2 Column Purified DNA

The genomic DNA from the bacterial strains was isolated from bacterial pellets that were obtained by first streaking a loop full of bacterial freeze stock onto LB agar plates and incubation the plate overnight at 37°C. Single colonies (5-6) were picked from the LB plate and suspended in 5 ml of LB media prior to over night incubation at 37°C with agitation of 225 rpm. The over night culture, which was in the stationary phase was diluted (2 ml of culture into 18 ml of LB media) and incubated for 5 – 6 hrs at 37°C with agitation. The bacterial solution was centrifuged at 4000 rpm for 10 minutes and the supernatant discarded prior to storage at -20°C until required for used.

Isolation: The isolation of DNA from the cell material pellet was performed with the QIAGEN Genomic DNA kit (QIAGEN, Hilden, Germany) that was used according to the manufacturer's instructions. The QIAGEN genomic DNA kit is designed for direct isolation of chromosomal DNA varying in size from 20 to 150 kb and is based on optimized buffer systems for lysis of cells and or nuclei. Upon lysis the genomic DNA is bound to the QIAGEN Anion-Exchange resin under appropriate conditions. The impurities are washed away by a medium-salt wash and the genomic DNA is eluted in a high salt buffer, concentrated and desalted by isopropanol. Briefly the thawed bacterial pellet was resuspended in 3,5 ml of buffer B1 to which 7 µl of 100 mg/ml of RNase had previously been added. A volume of 80 µl of lysozyme stock solution (100 µg/ml) and 100 µl of proteinase K stock solution was added to each sample and the tubes were incubated at 37°C for 30 minutes. To each tube, 1, 2 ml of buffer B2 was added and mixed by inverting the tube 7 times before incubation at 50°C for another 30 minutes. The QIAGEN Genomic-tip 100G was equilibrated by allowing 4 ml of buffer QBT to flow through the column by gravity flow. The genomic DNA was applied to the equilibrated QIAGEN genomic tip and allowed to flow through the resin by gravity. The resin was washed twice with 7.5 ml of buffer QC prior to elution of the genomic DNA with 5 ml of pre warmed (50°C) buffer QF. The DNA was precipitated in 3.5 ml of isopropanol into a white mass that was then washed in 2 ml of 70% ethanol. The DNA was allowed to air dry for 10 minutes and dissolved in 100 µl TE buffer. The procedure used in the isolation of plasmid DNA is described in section 5.2.

2.3 DNA quantification

The concentration of the eluted genomic DNA was determined by spectrophotometry with the Nanodrop® ND-1000 (NanoDrop Technologies INC, Wilmington, DE, USA). The quantity and purity of the DNA was determined by measuring the absorbance at 260 nm with pure DNA having a 260/280 optical density ratio not exceeding 1.8.

3.0 PCR

The amplification of DNA fragment by polymerase chain reaction (PCR) allows the use of a mixture containing a heat stable DNA polymerase, four deoxyribonucleoside tri phosphates (dATP,dCTP, dGTP, dTTP); a set of primers that are each complimentary to the DNA fragment and that acts as the precursor for DNA synthesis and the DNA template (Mullis 1990). The Taq polymerase is a heat stable enzyme isolated from the thermophilic bacterium *Thermus aquaticus* that has been widely used in DNA amplification and cycle sequencing. Its ability to remain stable at temperatures above 90°C allow for the PCR reactions to be performed at high temperatures thus increasing the specificity, yield and length of the amplified products (Innis et al. 1988). The PCR reaction is generally composed of three steps namely denaturation, annealing and elongation or extension. During denaturation, the reaction mixture is heated up to 94-96°C for approximately 5 minutes in order to break the hydrogen bonds that hold the DNA strands together. Once the strands are separated, the step which is annealing begins where the temperature is lowered to approximately 5°C lower than the melting temperature of the primers. This allows for the primers to attach to the single stranded template thus providing the polymerase with a precursor. During the elongation the DNA polymerase attaches to the already annealed primers and uses the dNTPS to synthesize the new strand as it moves along the template strand. The elongation step is run at a temperature that is optimal for the polymerase (68-72°C) and the elongation time is dependant on the length of the fragment to be amplified with 1 minutes normally corresponding to 1000 base pairs. A typical PCR reaction is run for 25- 35 cycles. In order to monitor the performance of the PCR, both positive and negative controls should be included in the run as the first confirms that the PCR works and the later confirms that the amplification is free of contamination. In this study two different polymerases were used in the amplify DNA fragments of varying sizes in the PCR PTC-200 thermal cycler (BIO-RAD, Norway), that is, DyNazyme II DNA polymerase and Phusion High Fidelity DNA polymerase both from Finnzymes, Finland.

3.1 DyNazyme II DNA polymerase

The amplification of genomic DNA segments in reactions where the PCR products was smaller than 3 kb (and for non-cloning purposes) was achieved in a reaction volume of 25 µl with the DyNAzyme™ II PCR Master Mix (Finnzymes, Finland). The reaction mix contained 10 µl DyNAzymes™ II DNA Polymerase (containing 0.04 U/µl DyNAzyme II DNA Polymerase, 2x optimized DyNAzyme Buffer and 400 µM of each dNTP), 7 µl PCR water, 1 µl of each primer (50 µM), and 1 µl template. The primers, annealing temperatures, product sizes and the number of cycles for each reaction are given in table 3.

3.2 Phusion High Fidelity DNA polymerase

The Phusion master mix (Finnzymes, Finland) was used to obtain PCR products that were larger than 3 Kb and for PCR products required for subsequent DNA ligation steps. The reaction mix was run in a volume of 30 µl which contained 18.5 µl PCR water, 6 µl 5X Phusion buffer, 0.3 µl 10 mM dNTPs, 0.3 µl of each primer (50 mM), 1 µl template , 3 µl DMSO 0.3 µl $MgCl_2$ and 0.3 µl of the Phusion polymerase. The PCR program started with an initial denaturation at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 30 seconds. The final extension was conducted at 72°C for 30 seconds and the PCR product was kept at 4 °C until needed for further applications. Table 3 gives the primers and the PRC programs that were used in the study.

Table 3: List of primer set, expected product size and the PCR programs used in the PCR reaction.

Primer set	Expected product size(bp)	PCR Programs
IntF2/Orf3R	4000	98 °C- 30 sec, 98 °C- 10 sec, 58 °C- 10 sec, 72 °C- 30 sec, repeated 30 times, 72 °C- 30 sec, hold at 4 °C
IN5'CS /IN3'CS	Variable	94 °C- 5 min, 94 °C- 1 min, 58 °C- 1 min, 72 °C- 5 min, repeated 34 times, 72 °C- 10 min, hold at 4 °C
aadBF/aadBR	1000	94 °C- 5 min, 94 °C- 1 min, 58 °C- 1 min, 72 °C- 5 min, repeated 34 times, 72 °C- 10 min, hold at 4 °C
ImpU/IMPL	587	94 °C- 5 min, 94 °C- 1 min, 58 °C- 1 min, 72 °C- 5 min, repeated 34 times, 72 °C- 10 min, hold at 4 °C
UpF2/downR	Variable	98 °C- 30 sec, 98 °C- 10 sec, 58 °C- 10 sec, 72 °C- 30 sec, repeated 30 times, 72 °C- 30 sec, hold at 4 °C
UpF3/down3R	Variable	98 °C- 30 sec, 98 °C- 10 sec, 58 °C- 10 sec, 72 °C- 30 sec, repeated 30 times, 72 °C- 30 sec, hold at 4 °C
UpF/GCS1Revcomp	1600	98 °C- 30 sec, 98 °C- 10 sec, 58 °C- 10 sec, 72 °C- 30 sec, repeated 30 times, 72 °C- 30 sec, hold at 4 °C
Sul-outF2/downR	1650	98 °C- 30 sec, 98 °C- 10 sec, 58 °C- 10 sec, 72 °C- 30 sec, repeated 30 times, 72 °C- 30 sec, hold at 4 °C
ACAIDupF2/ACAIDUpR	750	98°C- 30sec, 98°C- 10 sec, 58°C- 10 sec, 72°C- 30 sec, repeated 30 times, 72°C- 30 sec, hold at 4°C
ACAIDdonwF/ ACAIDdownr	700	98°C- 30sec, 98°C- 10 sec, 58°C- 10 sec, 72°C- 30 sec, repeated 30 times, 72°C- 30 sec, hold at 4°C

* The PCR product size is variable as it is dependent on the size of the gene cassettes

3.3 Purification of PCR products

The purification procedure is based on the use of a silica membrane assembly that binds the DNA in high-salt buffer followed by elution of the DNA with a low-salt buffer or water. The procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, and other impurities from DNA samples. The NucleoSpin® Extract II commercial kit (Macherey-Nagel, Germany) was used to purify the PCR products according to the manufacturers' instructions. The purification was applied to both PCR product and extracts from agarose gel. The purified product was eluted in 50 µl of the provided elution buffer.

Table 4: Primers used in this study.

Primer name	Nucleotide Sequence	Reference
Intf2	TCCGCCAGGATTGACTTGCG	This study
ORF3R	TCGCGAACCAAGACATCGC	This study
GCS1 RevComp	CGAGGCATTTCTGTCCTGG	Da Silva et al. 2002
Orf5-outF2	CCATGCCGCGCGAACGCAGGGGTGC	(Fournier et al.)
SeqintF2	TAGTGATTGCGCCAGGATTG	Da Silva et al. 2002
SeqOrfR2	AACGACGATTGCTGCTCACT	(Fournier et al.)
Orf5R	AAGTGTCGACGTGGGTGAAT	(Fournier et al.)
In5´CS Revcomp	CTTGCTGCTTGGATGCC	Da Silva et al. 2002
Integrase1F	ATGCGGCACCGATGGCCTTC	(Bissonnette and Roy 1992)
Qac 1R	CAAGCTTTTGCCCATGAAGGC	(Sandvang et al. 1997)
OrfReverse4	CGGTCTGCAAGTGATCTTGA	(Bissonnette and Roy 1992)
aadBForRight1	AGTCCAACTCCTCCATGA	This study
aadBForRight2	CGCAAGACCTCAACCTTTTC	This study
aadBForRight3	CGCCGACATTTCAACTATT	This study
aadBForRight4	ATCATCGTGCTTGCGTGTTA	This study
aadBRevLeft1	GCCGATGAAGTACCACCAGT	This study
aadBRevLeft2	ACTTGACTGCGAACCTGCTT	This study
aadBRevLeft3	CGTCATGGAGGAGTTGGACT	This study
aadBRevLeft4	GTGTAACACGCAAGCACGAT	This study
ACIAD3309down-F	CTTCTAGACTCTGTCGGTGCAGCGTATAGTCTATCC	This study
ACIAD3309down-R	AACTTGAGCGTCTTCAAGCATTTGAAGG	This study
ACAID3309upF	GTAAGTCTTCCCCAGCCTGCACG	This study
ACAID3309upR	AGAGCTCAAACCGCATATTATTTCCGTTAAAACACG	This study

3.4 Gel electrophoresis

The separation of macromolecules, proteins and/or DNA based on their size, electrical charge and their physical properties can be achieved by gel electrophoresis. The principle of this method lies in the different rates at which particles of different sizes and length migrate through a medium with pores (gel). Agarose is dissolved in boiling TAE buffer and allowed to cool to temperatures below 50°C in a gel cast. After solidification, the gel is loaded with the samples that are mixed with a loading dye (containing 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA) to prevent the samples from being lost in the running buffer. An electrical charge in applied to the gel and the particles in the samples migrate through the gel at different rates based on their particle size and the applied charge. Fragments of smaller size move faster and thus end up furthest in the gel whilst larger fragment tend to take a longer time to migrate through the gel (Johansson 1972). The varying locations of the samples in the gel are visualized by the addition of ethidium bromide, a stain the associates with double stranded DNA and is made visible by UV light (Sambrook et al. 1989).

4.0 DNA Sequencing

The nucleotide sequence of a fragment of DNA can be determined using the DNA sequencing method developed by Sanger and colleagues. The principle was based on the generation of DNA fragments using a DNA polymerase in the presence of deoxynucleotide- triphosphates and fluorescent- labeled dideoxynucleotide-TP which terminate the synthesis at nucleotide specific points along the target strand. A strand of DNA that is complementary to the template was synthesized by the extension of the primer that has attached to the template. During the extension, dNTPs were added or incorporated to form a fragment and this process continued until a dideoxynucleotide triphosphate (ddNTP)(Fuller 1997). The ddNTPs did not poses the 3'-OH group that is required for chain elongation and the fragments ends at this point. The fragments which vary in size were separated by electrophoresis on an acrylamide gel and the nucleotide order can be determined by scanning up the gel and analyzing the bands. The more recent methods employ a different fluorescent color emitter for each of the four reactions and use automated fluorescence detection machines (Strachan and Read 1999). All four reactions are then loaded into a single lane and a monitor detects and records the fluorescence signals that are emitted as the DNA passes through a fixed point in the gel.

The sequences of the integrons in the donor strains and in the transformants were determined by sequencing the purified PCR products obtained from primers IntF2, aadBF, downR and OrfR3. The sequencing reaction was performed with ABI big dye 3.1v cycle sequencing terminator reactions (Applied Bio systems) in a total volume of 20 µl. The reaction contained 4µl of purified PCR product, 4 µl of sequencing buffer and 4 µl of the primer at a concentration of 10mM. The sequencing PCR program was run in 25 cycles for 3 min at 96ºC, 15 s at 96ºC, 10 s at 50ºC, and 4 min at 60ºC. The ABI3130XL 20 genetic analyzer (Applied Biosystems) was used to read the sequences and the obtained sequence was analyzed and manually edited by the Sequencher v.4.2.2 program (GeneCodes, USA). All generated sequences were compared to and blasted against previously published sequences that are available at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

The nucleotide sequence of the regions outside the gene cassettes in the donor strains which was unknown was obtained by primer walking (Ray et al. 2009). In this method, the known sequences of gene cassettes which are positioned in the integron were used to design specific primers for each strain that were diverging from gene cassette and into the conserved regions of the integron. The flanking regions around the integrons were also obtained with this method. The method used is based on the description given by (Parker et al. 1991).

4.1 Primer design

The amplification of a segment of DNA requires primer pairs that would attach to the complementary template strand and serve as elongation starting points for the DNA polymerase. The need for specific primers led to the design of specific primers that could be implored in this study. The primers that were used in this study were partially designed with the Primer3 software from http://fokker.wi.mit.edu/primer3/input.htm. The designed primers were analyzed with the Oligoanalyzer program from integrated DNA Technologies Inc. The primer sequences that were used in this study are given in table 4.

5.0 Construction of *A. baylyi* carrying a class 1 integron

The attempts to construct an *A. baylyi* strain that contained the class 1 integron of clinical origin involved several transformation assays using a variety of methods.

5.1 Construction of plasmid vectors

The integration of a class 1 integron into the model organism *A. baylyi* by homologous recombination required the use of a vector (pGT41). The fitness neutral chromosomal location of the ACAID3309 (putative lipase) gene (P. Johnsen, unpublished) was chosen as the insertion site. DNA segments containing regions upstream and downstream of the targeted insertion site (referred to as "up flank" and "down flank" subsequently) were amplified by PCR employing primers with suitable 5' tails and ligated to pGT41 and its derivatives. This would then enable the insertion of the integron at the defined target site by homologous recombination. The plasmid pGT41 (shown in figure 4) is a medium copy number plasmid (approximately 50 copies per cell; Klaus Harms, personal communication) that contains a chloramphenicol gene (*cat*), a 3'- deleted ampicillin gene (*bla-4*) conferring low resistance and a 3' deleted (defective) kanamycin resistance gene (*nptII-7*) and a gene that impairs sensitivity to sucrose (*sacB*). The presence of the inactive versions of the kanamycin resistance gene (*nptII-7*) which lacks the last 7 nucleotides on the 3' end, and the ampicillin resistance gene (*bla-4*) which lacks the last 4 nucleotides on the 3'end provide the possibility to positively select for the markers that can be activated by insertion of DNA fragments with the specific sequence. These missing nucleotides are provided by specific 5'- extensions of primers used for PCR amplification (see Table 4). The Up and down flanks that were inserted into the 5' ends of the *nptII-7* and *bla-4* genes, respectively were designed to reactive these genes and thus serve as confirmation of insertion in the right position and orientation. In addition to providing confirmation of correct insertions by gene activation, this plasmid also has the advantage of containing the *sacB* gene which confers intolerance to sucrose. This cassette provides an easy yet elaborate method for the selection of transformants.

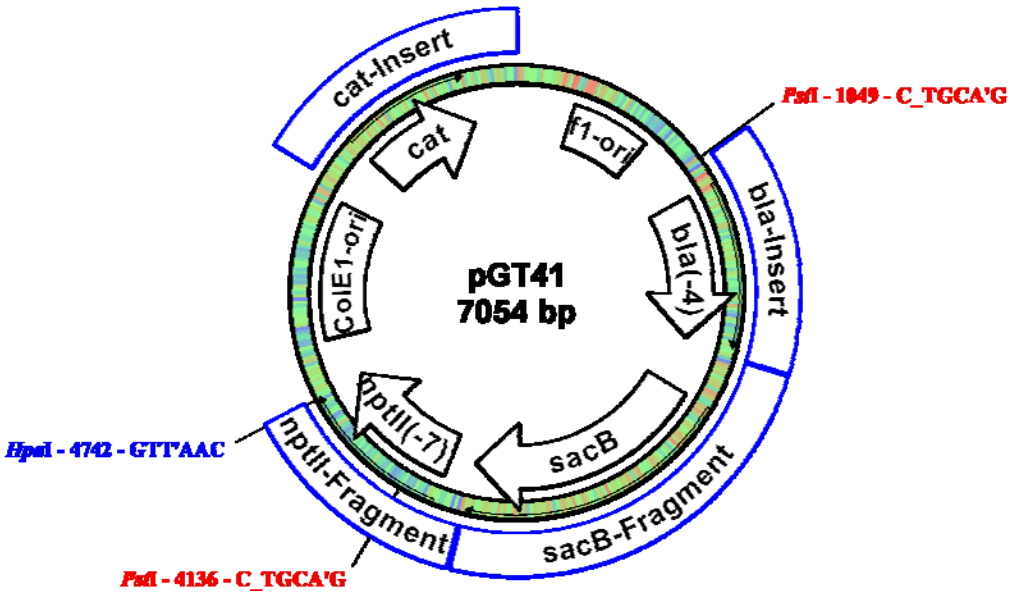


Figure 4: A schematic representation of the plasmid pGT41. The arrangement of the genes and the restriction sites of *Pst*I and *Hpa*I are shown. The *bla-4*: an inactive version of the ampicillin gene; *sacB*: a gene conferring sensitivity to sucrose; *nptII-7*: inactive kanamycin resistance gene; ColE1- ori; *cat*: gene conferring chloramphenicol resistance. The plasmid has a size of 7054 bp. The f1-ori is the phage f1 origin for production of single stranded DNA (and has no relevance to this study).

5.2 Plasmid DNA isolation

The isolation of the plasmids DNA was achieved with the QIAGEN Plasmid Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. The QIAGEN plasmid purification protocol is based on a modified alkaline lyses procedure, which is followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under low-salt and pH conditions. The impurities are removed by a medium-salt wash and the plasmid DNA is eluted in a high-salt buffer and isopropanol is used to concentrate and desalted the plasmid DNA.

A loop full of SF8 *rec*A (Romanowski et al. 1993) provided by Klaus harms was streaked LB plate containing 25 mg/l chloramphenicol was inoculated into a starter culture of 25 ml LB containing 25 mg/L chloramphenicol. The culture was incubated at 37°C with vigorous shaking for approximately 12 hours. The bacterial cells were harvested by centrifuging the starter culture at 6000 x g for 15 minutes at 4°C. The bacterial cells were re-suspended in 2 ml

buffer P1 and upon complete suspension of the pellet 2 ml buffer P2 was added and mixed by inventing the tube. The mixture was incubated at room temperature for 5 minutes prior to the addition of 2 ml pre chilled buffer P3, thorough mixing and centrifuging at maximum speed for 10 minutes. The supernant was promptly removed and transferred into a QIAGEN tip 20 that had previously equilibrated with 1 ml buffer QBT. The QIAgen tip 20 was washed with 2 ml buffer QC twice and the DNA was eluted from the resin with 800 µl buffer QF. The DNA was precipitated by adding 560 µl room temperature isopropanol and centrifuging at 10000 rpm for 30 minutes. The supernant was discarded and the pellet washed in 70% ethanol by a 10 minute centrifuging step. The ethanol removes the precipitated salts and allows for the DNA easier to re-dissolve. The pellet was air dried for 10 minutes and re dissolved in 100 µl TE buffer. The concentration of the isolated plasmid DNA was determined by UV spectrophotometry at 260 nm using the Nanodrop® ND-1000 as described in section II – 2.3.

5.3 Treatment with restriction endonucleases

The plasmids that were isolated in this study were digested with various restriction enzymes to generate fragments of varying sizes and length. Table 5 gives the name, restriction sites, buffer conditions and fragments sizes generated by each restriction enzyme. All the digestion reactions were performed at 37°C with 1 hour incubation prior to enzyme inactivation at 65°C for 20 minutes. The digested plasmids were run on 1% agarose gel to verify the sizes of the generated fragments and low mass ladder was used to estimate the DNA quantity.

5.4 Ligation

The ligation of the digested plasmid to either provided DNA fragments or to itself (as was the case for pTM 3) was facilitated by T4 DNA ligase, an enzyme purified from *E.coli*, that catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA and joins blunt ends and matching cohesive ends (Sambrook and Russell 2001). The reactions were carried out in a final volume of 10 µl containing digested vector, insert (vector and insert where kept at approximately equimolar concentrations) 400U T4 DNA ligase and 1 µl 10X ligation buffer (both from Biolabs Inc. New England) according to the manufacturers' protocol. The ligation mix was incubated at room temperature for 12-16 hrs and the ligation products were visualized (typically 10% of

the ligation mix was used) on a 1% agarose gel by electrophoresis at 80V for 1hour.

Table 5: A list of indicating the plasmids, restriction enzymes and restriction sites used in this study.

Plasmid	Restriction Enzyme	Restriction site
pGT41	*KspA* I	5′GTT↓AAC3`
		3`CAA↑TTG5`
pTM1	*Oli* I	5′ CACNN↓NNGTG3’
		3’ GTGNN↑NNCAC5’
pTM1-3	*Ecl*136 II	5’ GAG↓CTC3’
		3’ CTC ↑GAG5’
pTM1	*Xba* I	5′T↓CTAGA3`
		3′AGATC↑T3
pTM1	*Xho* I	5`C↓TCGAG3′
		3′GAGCT↑C5′
pTM3	*Sac* I	5′GAGCT↓C3′
		3′C↑TCGAG3′
pTM3	*Xmn* I	5′GAANN↓NNTTC3′
		3′CTTNN↑NNAAG5
pTM4	*Ecl*136II	5’ GAG↓CTC3’
		3’ CTC ↑GAG5’

5.5 Preparation of electro-competent cells

Fresh overnight bacterial cultures of either *E.coli* and or *A. baylyi* which was prepared from one colony of bacteria with a volume of 1/100 was added to 1 liter LB media and the cells were grown at 37°C with vigorous shaking until the cell titer reached 2 x 10^8/ml determined by hemocytometer (Neubauer). The cells were chilled on ice for 15 minutes prior to a centrifuging step at 4000 x g at 4°C for 15 minutes. The supernatant was discarded and the pellets re-suspended in 1 liter of cold water. The suspension was centrifuged and the pellet re-suspended in water prior to another centrifugation step and re-suspension in 20 ml cold 10% glycerol. The glycerol and cells mixture was centrifuged and re- suspended in a final volume of 2 ml in cold 20% glycerol. The cells were divided into aliquots of 40 µl and frozen in nitrogen prior to storage at -80°C.

5.6 Construction of *A.baylyi* strain carrying class 1 integron by electroporation

Competence in bacterial populations is a prerequisite for the uptake of DNA from the bacteria's environment by the process of transformation. Competence may be constitutively expressed or only expressed at certain stages of the life cycles of a bacterial species. Although competence is widely spread among bacterial populations, it only occurs in a small percentage of the known bacterial species. It is for this reason that methods such as electroporation that enhance the bacteria's ability to take up external DNA have been developed (Wu and Seitaridou 2007). In general this method involves the application an pulsed electric current that exceeds its dielectric strength of the cell membrane for short period of time thereby creating pores (Chu et al. 1987). These pores are resealed after a short period of time but prior to the resealing; the external DNA is able to enter the bacterial cell (Wu and Seitaridou 2007). The introduction of plasmids that carry desired traits into *E. coli* by electroporation has lead to massive progress in molecular biology and is remarkably efficient (Hanahan et al. 1995).
In this study, the plasmid pGT41 was used to construct plasmid (pTM4) that carried the desired traits (the class 1 integron, flanked by DNA sequence that was homologous to the *lifO/lipB* region of *A.baylyi*) and this was introduced in *E.coli* by electroporation.

The plasmid pGT41, after digestion and ligation with the desired DNA fragment was introduced into *E.coli* competent cells (strain SF8 *recA or* EC100) by electroporation. The method used was previously described by Dower and colleagues (Dower et al. 1988). The

competent cells were thawed on ice and 1µl of the ligation mix was added to each tube containing 40 µl of the cells. The Gene Pulser II (BIO-RAD, Norway) was set at 2.5 kV, 200 Ω, 25 µF and 2 mm cuvette. The mixture of the competent cells and the DNA was transferred into a pre-chilled electroporation cuvette and the cuvette was placed into the safety chamber. The contents of the cuvette were pulsed once, producing a pulse with a time constant of between 4.3-4.7 msec. The electroporated cells were transferred from the cuvette by adding 1 ml of LB medium to the cuvette and quickly re-suspending the cells with a pasteur pipette. The cells were transferred to a new glass tube and incubated at 37°C for 1 hour with aeration. The suspension was centrifuged at 5000 x g for 5 minutes and the supernatant was discarded prior to the re-suspension of the pellet in 1 ml of fresh LB medium. The selection for transformants was achieved by plating typically 200 µl on LB plates with selective medium and incubation at 37°C overnight.

5.7 Splice PCR

The construction of recombinant DNA segments by PCR as described by Metzgar et al (2004) in the method termed splice PCR, provides a means of generating variable DNA fragments that can be used as donor DNA during transformation assays (Metzgar et al. 2004). In this method, the fragments of interest (which in the case of this study were the integron and the up and down flanks) are PCR amplified separately with primers that have 5' overhangs which are complementary to the fragment they are at be joined to. The up flank is a fragment of the ACAID3308 gene which encodes for the chaperon *lifO* and the down flank is a fragment of the ACAID3309 gene which encodes the putative lipase(Barbe et al. 2004). The obtained products are then re-amplified in a second round of PCR with only the outer most primers thus producing a "fused PCR product" that may be used as donor DNA in transformation assays.

The fusion of the flanking regions (up flank and down flank) to the integrons in order to facilitate homologous recombination was achieved by PCR amplifying each of the three fragments individually prior to fusion of the PCR products in a PCR reaction containing the most outward primers. The fusion was performed in steps with the first step being the individual amplification of the up flank (with primers UpF2/UpR2), the integron with primers IntF2 and Orf3R and the down flank with primers DownF and DownR. The second step was to run a PCR reaction with primers UpF2 and Orf3R which then created a fusion fragment of

the up flank and integron. This fused fragment and the down flank PCR product were used as template for the third round of PCR with primers UpF2 and DownR. The final fragment containing the UP flank, the integron and the down flank were verified by gel electrophoresis and used as a DNA source in a transformation assay with ADP1 as the recipient. The transformation assay was performed as previously described in section 2.8 and the obtained transformants were subjected to phenotypic and genotypic testing.

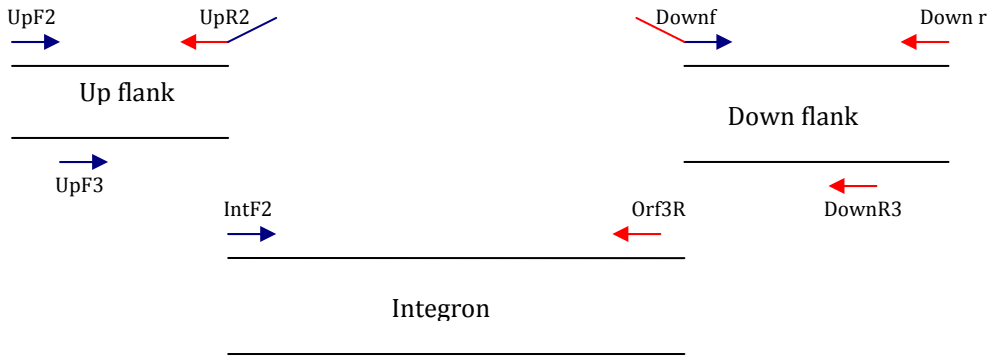


Figure 5: A figure illustrating the priming sites of primers used in the fusion PCR (Splicing PCR) used to generate integron flanked by regions that are homologous to the *lipA/lipB* segments in ADP1.

5.8 Natural transformation of *A. baylyi* (liquid transformation)

The liquid transformation was preformed as described by de Vries and Wackernagel (De Vries and Wackernagel 1998) with some modifications. Briefly the isolated bacterial colonies that were grown only selective media were used to prepare an overnight culture. The bacterial cells where harvested in log phase (approximately 1.0 X 10^9/ml) and were immediately used or immediately frozen at -80 °C as concentrated stock (1 x 10^{10}/ml). For transformation, the cells were diluted to 2.5 X 10^8 in fresh LB prior to the addition of the donor DNA (PCR product and/or linearised plasmids). The mixture was incubated at 37°C with 225 rpm aeration for 2 hours prior to centrifugation at 13000 rpm for 10 minutes. The pellet was re suspended in 0.9 ml LB and plated on selective plates. The plates were incubated at 37°C for 16-20 hrs. The resulting colonies were screen phenotypically by growing them on selective medium and any transformants the retained resistance to the selective medium were screened

by PCR to verify the insertion of the integron in the correct location and in the right orientation. The figure 6 below illustrates the insertion site of the integron in ADP1 and the primers used in the PCR screening process.

5.8.1 Construction of strain Ab64.T1b

The strain Ab64.T1b was constructed by ligating the PCR amplified integron from Ab64 (using primers IntF2/Orf3R) to the linearised plasmid pTM4 and using this ligation mix (after further linearization) as the donor DNA. The assay consisted of 1.5 ml fresh LB, 375 µl competent cells and 10 µl of PCR product.

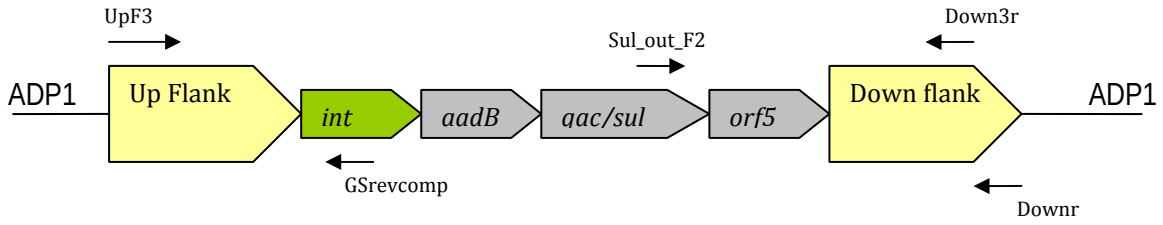


Figure 6: A schematic representation of Ab64.T1b after the insertion of the integron from Ab64 into ADP1. The diagram shows the position of the primers used in verifying the position and orientation of the inserted integron. The *int* encodes for the integrase; *aadB*: kanamycin resistance cassette; q*ac/sul*: quaternary ammonium and sulphonamide gene cassette and *orf5* encodes for a protein of unknown function.

The assay was performed as described in section 5.8 above and LB plates containing kanamycin (50 µg/ml) were used to select for transformants. The transformants that conferred resistance to kanamycin 50 µg/ml were further screened by PCR using the primers that are listed in the figure 6.

5.8.2 Construction of strain Ab64.T1b *int::cat*

The construction of the strain containing an inactive integrase gene was achieved by natural transformation by the plasmid pACYC177–int-cat (constructed by Klaus Harms and shown in figure 7). The plasmid carries a truncated integrase gene which has been inactivated by the insertion of a chloramphenicol resistance gene *cat*. The plasmid was isolated from single colonies grown in the presence of 25 µg/ml chloramphenicol and isolated using the QIAGEN Plasmid Purification Kit. The isolated plasmid was linearised by restriction enzyme digestion with *Hind*III and used as a source of the inactive integrase in a transformation assay with strain AB64.T1b as the recipient. The transformation assay was conducted as described in section 5.8. The transformants were selected on chloramphenicol 25 µg/ml or 10 µg/ml and the obtained single colonies were subjected to phenotypic and genotypic tests.

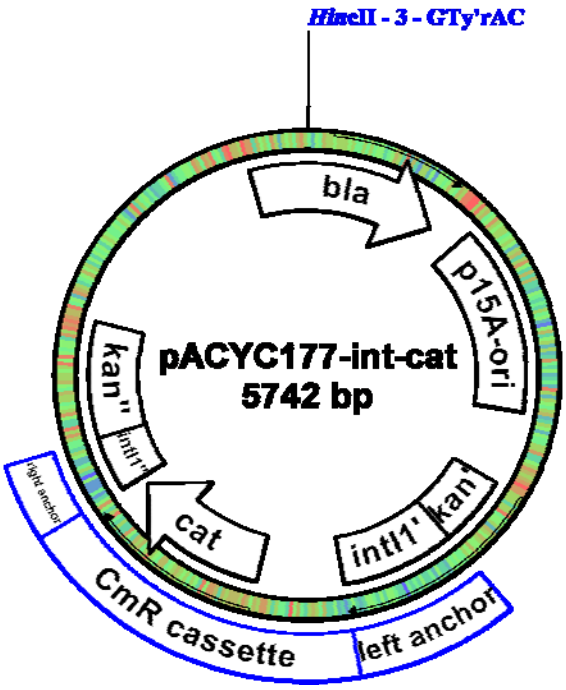


Figure 7: A schematic representation of the plasmid pACYC177-int-cat illustrating the *Hind*III restriction site; the inactivated integrase gene *intI*1'; the inserted chloramphenicol gene *cat*; the ampicillin resistance gene *bla*; and the dirupted kanamycin resistance gene *kan*'.

5.9.3 Construction of ADP1.*nptII.sacB*

The strain ADP1.*nptII.sacB* which contained the selectively neutral genes *nptII* and *sacB* in the selectively neutral locus *LifO/LipB* was constructed with ADP1 as the recipient and strain. The plasmid pTM2 which contained the gene cassettes *nptII* and *sacB* was used as donor DNA in a transformation assay performed as described in section 5.8. The transformation assay consisted of 25 µl ADP1 competent cells dissolved in 1 ml fresh LB and 1 µl of the *Xho* I digested plasmid. The transformants were selected for on LB plates containing 50 µg/ml kanamycin.

6.0 Cassette recruitment

In nature, bacteria are known to acquire and accumulate resistance genes there by rendering them multi resistant to antimicrobial agents. In order to assess the biological cost and stability of newly acquired integrons and cassettes, a gene recruitment study was conducted. In this study, competent cells of the strain Ab64.T1b which carries a newly acquired integron were prepared as stated in section 5.5. The competent cells were frozen and stored at -80°C until required for use. The integrons in the donor strains, Ab65FFC, K34-74 and A3-54 were PCR amplified using the integron specific primers IntF2 and Orf3R and used in liquid transformation (as described in section 5.8). The transformed cells were selected for by growing the Ab65FFC transformants on ampicillin (100 mg/L) plates, the K34-74 transformants on kanamycin (50 mg/L) with meropenem (25 mg/L) and the A3-54 transformants on Ampicillin (100 mg/L) with meropenem (25 mg/L). The transformants were further screened genotypically by PCR with integron and cassette specific primers in order to verify their identity.

7.0 Antimicrobial susceptibility and resistance test

The susceptibility and resistance levels of the *A. baumannii* strains Ab 64 and Ab 65FFC, the *P. aeruginosa* strains K34-74 and A3-54 and all obtained transformants were determined with the Kirby-Bauer (Disc diffusion) method and the E-test respectively. Theses studies provide an overview of the resistance patterns of the donor, recipient and transformants thus providing a basis for observing alternations or changes in the resistance levels between the test strains.

7.1 Disc diffusion method

The method is based on the use of filter paper discs that are impregnated with antibiotics of known concentrations which when placed on the agar diffuse from the disc and into the agar surrounding the disc. The antibiotics that are provided will inhibit growth around the discs in what is termed the "zone of inhibition" if they are effective against the bacteria present on the agar. The test was performed according to the procedure provided by the K-res (Reference Centre for Detection of Antimicrobial Resistance, UNN, Norway). Briefly, a loop full of the freeze stock solutions was streaked on LB plates and incubated overnight at 37°C prior to the selection of single colonies that were suspended in 5 ml 0.9% NaCl solution. The solution was adjusted to the turbidity of 0.5 McFarland standards and plated onto Mueller- Hinton plates (BioChemika, Switzerland) before the diffusion discs that corresponded to the antibiotic resistance gene cassettes contained by the integron in each strain were applied to each quarter of the plates. The discs used in this study were ceftazidime 30 μg, kanamycin 30 μg, meropenem 10 μg, ampicillin 2 μg and gentamicin 10 μg.

7.2 E- test

The determination of resistance level in bacteria has been conducted in several ways, however the E-test remains among the most commonly used method in both clinical and research settings. The E-test strips are contains known concentrations of an antibiotic agent that are present in a gradient along the strip with the lowest value being 0.16 μg/ml and the highest value being higher that 256 μg/ml. The diffusion of the antibiotics from the strip and into the agar allow for the quantitative measuring of the minimal inhibitory concentration (Brown and Brown 1991).The E-test was used to determine the minimal inhibitory concentrations (MICs) of the strains used in this study. A bacterial suspension with the turbidity of 0.5 McFarland of each bacteria strain was obtained by growing the bacteria at 37°C on LB plates with appropriate antibiotic for each strain and then suspending single colonies in 0.9% NaCl. The bacterial suspension was then spread on Mueller-Hinton (Fluka, BioChemika, Switzerland) and the E-test strip was placed at the centre of the plates prior to incubation at 37ºC for 20 hrs. The test strains were tested with E-test strips containing ceftazidime, cefotaxime, gentamicin, kanamycin and streptomycin. The minimal inhibitory concentrations of the recipient strain, ADP1 and all the obtained transformants were also determined using the above mentioned antibiotics.

8.0 Fitness competitions

The biological cost, that is fitness cost of an acquired trait is considered one of the main parameters that determine whether or not the trait will be maintain and/or spread among the bacterial population. Relative fitness which is defined as the relative rates at which resistant and sensitive bacteria grow and die (compete) in hosts and environments (Bennett AF and Lenski 1993) was used in this study to determine the fitness cost of the newly acquired traits. The study consisted of 24hrs direct competition between the original *A. baylyi* strain (ADP1) and each of the three strains (Ab 64.T1b, Ab64.T1bInt::cat, ADP1.*nptII.sacB* that contained the newly acquired class 1 integron. The strain Ab64.T1B carried the fully active class 1 integron that conferred resistance to aminoglycosides where as strain Ab64.T1bint::cat carried an integrase inactive insert of the same integron. The inactivation of the integron through the insertion of the chloramphenicol (*cat*) gene provided a means of assessing the cost of the integron and more specifically the integrase gene. The strain ADP1.*nptII.sacB* was included in the study in order to assess the cost of neutral cassettes inserted in the selectively neutral locus *LifO/lipB*.

8.1 Relative Fitness

The relative fitness was estimated by pair wise competition experiments which were carried out between the wild type strain *A. baylyi* ADP1 and the transformant strains Ab 64.T1b, Ab64.T1bint::cat, ADP1.*nptII.sacB*. The fitness competitions were run in six parallels from independent starter cultures which were prepared from single colonies that were grown on selective media prior to inoculation in 3ml of S2 media with 2% lactic acid. The cultures were incubated overnight at 37°C with aeration at 225 rpm. The overnight cultures were diluted 1:10 in 0.9% NaCl giving a final volume of 3 ml. The OD of the diluted solutions was adjusted to 0.5 prior to mixing the two competitors at a ratio of 1:1 (150 µl of each) in 2.7 ml of S2 minimal medium supplied with 0.1 µg/ml DNAse. A volume of 100 µl was immediately transferred to a 1.5 ml eppendorf tube and used to make 100 fold serial dilutions in 0.9% NaCl ranging from 10^{-1} to 10^{-4}. A volume of 100 µl was plated on both antibiotic free plates and antibiotic supplemented LB plates prior to counting the number of colonies obtained (N_0) after 48hrs of incubation at 37°C. The competition culture was incubated at 37°C with aeration at 225 rpm for 24 hrs after which 100 fold serial dilutions of the over night culture were prepared ranging from 10^{-1} to 10^{-6}. The serial diluted suspension were plated on both

selective and non selective plates and incubated at 37°C and the number of colonies obtain after 48 hrs incubation were counted and recorded as the final density (N_1). The initial and final cell density of the competitors was calculated by subtracting the number of colonies on the selective (LB + antibiotic) plates from the non selective (LB) plates. The fitness cost was calculated using the equation derived from Bennett and Lenski (1993):

$$M = \ln(N_1/N_0)$$

$$W = m1/m2$$

Where

m is the Malthusian parameter of each strain

N_1 is the final cell densities in direct competition (cells per ml)

N_0 is the initial cell densities in the direct competition (cells per ml)

w is the relative fitness of each genotype

m1 and m2 are the Malthusian parameters of resistant strains and susceptible strains respectively (Bennett AF and Lenski 1993).

9.0 Stability of integrons

The stability of the cassettes in the integron containing strains K34-74, A3-54, Ab 64, Ab 65FFC and in transformants Ab64.T11b and Ab64.T1b.Da were assessed over a period of 10 days. In order to obtain single colonies, the freeze stock solutions of each strain were streaked out on LB plates and incubating at 37°C over night. The single colonies were emulsified in 10 ml of pre-warmed LB media and incubated at 37°C with aeration to produce an overnight culture. A fresh culture solution containing 100 µl of the overnight culture solution and 9.9 ml of fresh LB media prepared and incubated overnight at 37°C with aeration at 225 rpm to produce the day 0 culture. Fresh cultures containing 100 µl from the day 0 culture and 9.9 ml of fresh LB and LB with the appropriate antibiotic were made daily for 10 days. Each overnight culture (100 µl) was serially diluted in 900 µl 0.9% NaCl until dilutions of 10^{-6} were obtained. The 10^{-6} dilution was plated on LB plates to yield the colonies that were replica plated on the appropriate selective plates. The colonies were picked one at a time and transferred to the selective plates (kanamycin 50 mg/L and sulphonamides 25 mg/L) and using the same tooth stick transferred to the LB plate which was used a control. The figure

below (figure 8) provides a schematic overview of the procedure that was performed daily for 10 days.

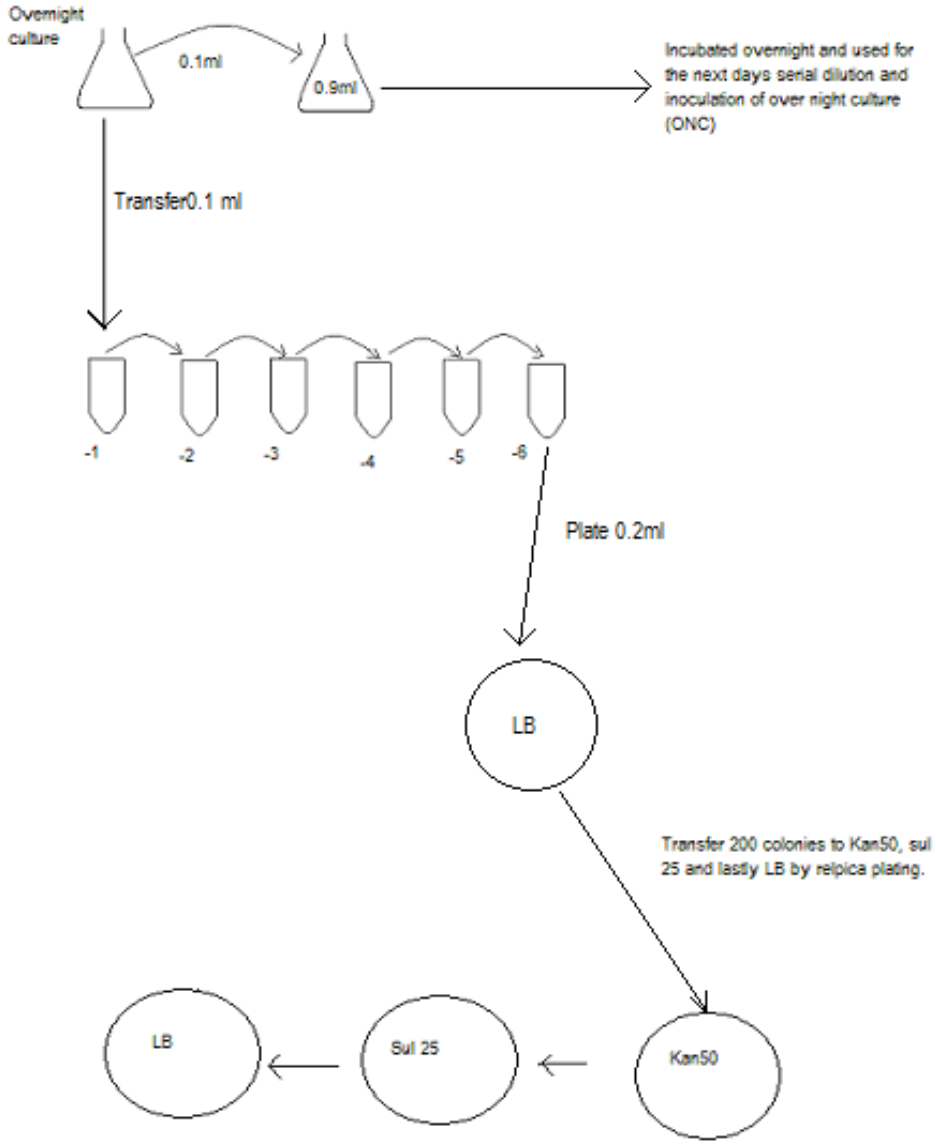


Figure 8: A schematic representation of the stability study of the test strain harboring class 1 integrons.

9.1 Cassette Excision

Gene cassettes are mobile genetic elements that are found as part of an integron. They can however occur naturally in their circular form either prior to integration by the integrase or after excision from the integron. The ability of the gene cassettes in a newly acquired integron to be excised was accessed by cultivating the strain Ab64.T1b in an environment that did not provide antibiotic pressure. The strain contains integron harboring the kanamycin resistance gene *aadB* was cultivated in LB at 37°C overnight and serial transfers of the over night culture into fresh LB medium were performed daily over a period of three days. The DNA in the over night cultures was extracted using the Qiagen DNA extraction kit and used as template in the excision PCR. The excision PCR was performed with the following primer sets.

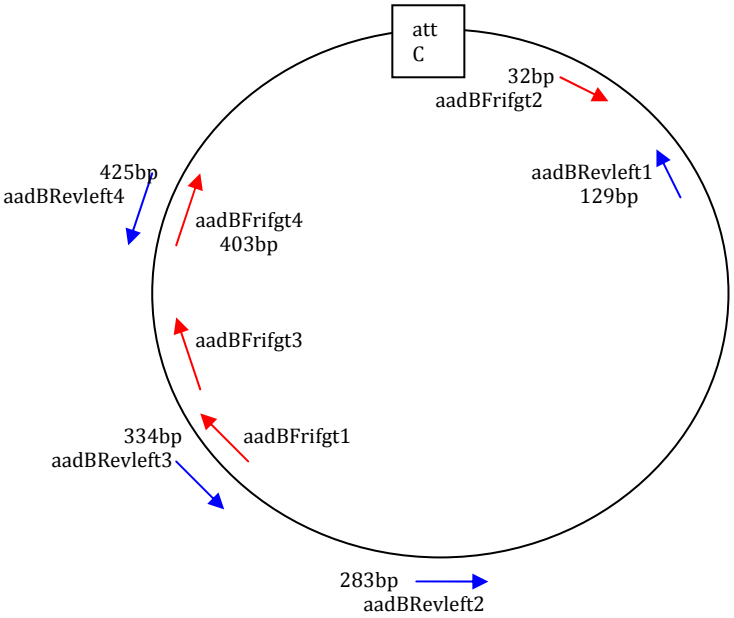


Figure 9: A schematic representation of the re- circularized gene cassette after excision from the integron and the location of the PCR primers.

SECTION III: RESULTS

1.0 The construction of an *A. baylyi* strain with a newly acquired class 1 integron

The construction of an *A. baylyi* strain that contained an integron required the insertion of a class 1 integron into the integron-free strain, ADP1. This was achieved by natural transformation with PCR- amplified integrons from the donor strains ligated to a specifically constructed plasmid which provides segments for chromosomal integron insertion by homologous recombination.

1.1 Amplification of class 1 Integrons

The integrons that are harbored in the bacterial strains used in this study were amplified using the primers and PCR programs listed in table 3. Primers IntF2 and Orf3R were used to amplify the entire integron which yielded PCR products of approximately 4000 bp both strain Ab64 and Ab65FFC. The obtained products corresponds to the expected size the reported sizes of the integrase, resistance genes, *qacEΔ1*, *sul1* and *orf5* (approximately 4000 bp, respectively). The sizes of the integrase, *qacEΔ1*, and *sul*1 genes were previously determined and these are reported to be 1014, 347 and 839 bp respectively (Tsakris et al. 2006). The *orf5/6* gene has been reported to be approximately 550 bp (Post and Hall 2009). The recipient strain ADP1 which was used as the negative control did not yield any PCR products, as expected. The primers IN5'CS/IN3'CS amplify the region located between the 5' and 3' conserved segments and in Ab64, this region corresponded to a region of approximately 850 bp where as in Ab65FFC the region was 1000 bp. The size difference of the PCR products is due to the different variable regions that are contained within the integrons with strain Ab64 containing the *aadB* gene which confers resistance to aminoglycosides and strain Ab65FFC containing the *bla-$_{IMP-5}$* which confers resistance to broad spectrum cephalosporins and carbopenems. The *aadB* gene has been reported to be approximately 750 bp (Cameron et al. 1986) but the PRC product (850 bp) obtained with primers IN5'CS/IN3'CS in this study was larger than the reported size as the primers amplify not only the gene cassette but parts of the 5' and 3' conserved segments. The PCR products obtained with the above mentioned primers in strain Ab65FFC was 1000 bp and this is in agreement with the results obtained by Da Silva (Da Silva G et al. 2002a) where identical primers were implored to amplify the conserved segment in the same strain.

The PCR amplified integrase, *aadB* gene, and flanking regions were sequenced to verify their identity and when compared to known sequences of the class 1 integrons and the gene cassettes, high degrees of identity. The integrase gene sequence that was obtained showed

99% identity to *A. baumannii* strain AB08-ColR where as the *aadB* showed 96% identity with another *A. baumannii* strain (AJ89190). The *sul1* and *orf5* regions when sequenced showed 98% identity with *A. baumannii* strain 3208 whilst the down flank in Ab64.T1b was 99% identical to A. baylyi ADP1. The generated sequences and BLAST results are given in appendix 1.The high similarity in sequence of the integrons contained in the test strains and those of published integron reaffirms previous finding that the structure of the 5' and 3' conserved segments are high conserved (Collis and Hall 1992b). In addition to sequencing the integrons in strain Ab64 and Ab65FFC, the flanking region of the integrons were also determined by primer walking.

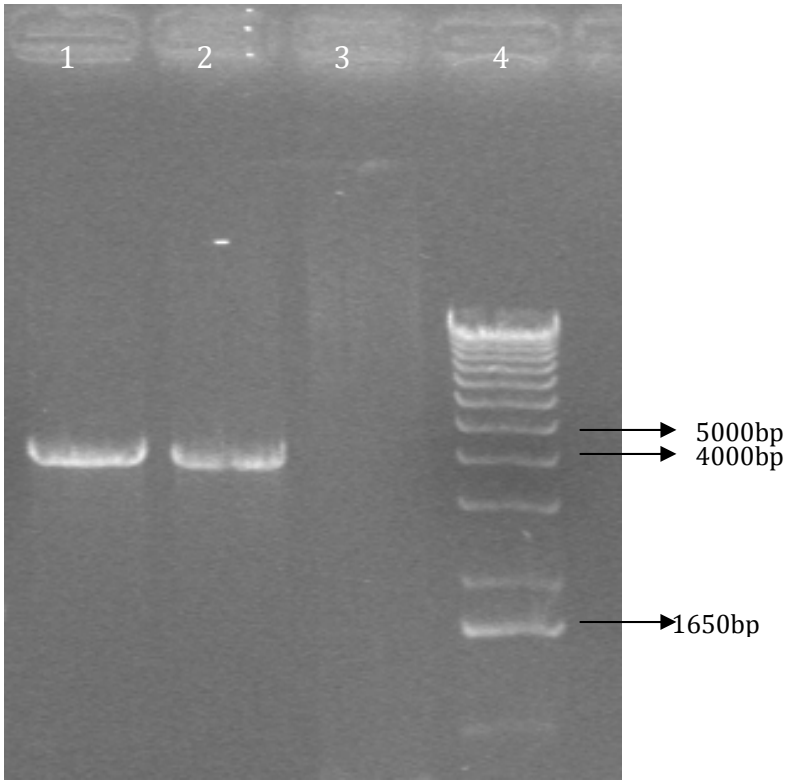


Figure 10: An image of 1% agarose gel showing the PCR amplified integrons from strain Ab64 and Ab65. Lane 1: Ab64 amplified with IntF2 and Orf3R, Lane 2: Ab65FFC amplified with IntF2and Orf3R; Lane 3: ADP1 amplified IntF2 and Orf3 as the negative control. Lane 4 contain the molecular weight ladder 1Kb plus.

1.2 Construction of the cloning vector

The construction of an integron containing *A.baylyi* strain required the use of a DNA vector (plasmid pGT41) into which the homologous regions (for subsequent chromosomal

integration, approximately 700 bp upstream and 750 bp downstream of the targeted integron insertion site) PCR amplified integron could be inserted. pGT41 was isolated from *E.coli* Sf8*recA* (Romanowski et al. 1993) with the QIAGEN Plasmid Purification Kit (QIAGEN, Hilden, Germany) and quantified according to section II: 2.3 (materials and methods).

The plasmid containing homologous regions with the *lifO* and *lipB* regions of ADP1 was generated in four steps resulting in four subsequent plasmids termed pTM1, pTM2, pTM3 and pTM4. In the first step, the upstream homologous segment (obtained by PCR using primers ACAID3309upF and ACAID3309UpR and *A.baylyi* ADP1 DNA as template) was inserted into the singular *Oli*I site of pGT41. Plasmid DNA was isolated from eight purified ampicillin resistant transformant colonies and analyzed by restriction analysis employing *KspA*I and *Pst*I. The restriction pattern was as expected (4.4 kbp, 33.1 kbp and 1.3 kbp). One clone was chosen and the plasmid was termed pTM1. Into this plasmid, the downstream homologous segment (obtained from primers ACAID3309downF and ACAID3309downR) was inserted into the unique *KspA* I site. The obtained clones were analyzed by restriction analysis employing *Xba*I, *Ecl136*II, *Xho*I. The *Xba*I restriction enzyme produced linear fragments of 317 bp, 2699 bp and 5224 bp where as digestion with *Ecl136*II yielded two linear fragments of 2705 bp and 5841 bp. The restriction enzyme *Xho*I only yielded one fragments of 8546 bp.

pTM3 was obtained from pTM2 by digestion with *Sac*I and relegation of the large fragment. This step removed the *sacB* and *nptII* genes and produced the singular *Ecl136*II restriction site for the plasmid insertion of integrons into the plasmid. The re-ligation of the rest of the plasmid produced a plasmid that did not contain the *nptII(-7)* and *sacB* regions. The excision of the two cassettes was verified by growing *E.coli* that contained this plasmid on LB media containing chloramphenicol and sucrose. The plasmid pTM3 still contained the ampicillin resistance gene *bla* hence limiting the use of this plasmid as selecting for ampicillin in Ab65FFC transformant would select not only for positive inserts of the integron but also those that contained the plasmid. To eliminate this limitation, plasmid pTM4 was generated by inactivating the ampicillin resistance gene (*bla*) by digesting it with *Xmn*I which created cuts in positions 22 bp and 1381 bp. The generated plasmid, pTM4 was 4482 bp in size, contained the ACAID3309 up and down flanks, remnants of the *bla* gene and the *cat* gene.

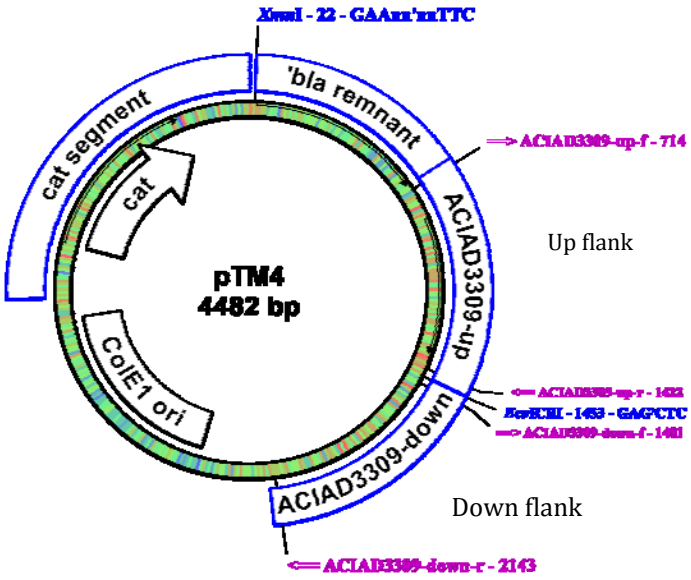


Figure 11: The plasmid pTM4 which was used as the vector for the integron. The figure illustrating the *Xmn*I and *E*cl136II restriction sites; the inserted flanking region ACAID3309-up and down, the priming sites for primers UpF, UpR, DownF and DownR; The chloramphenicol gene *cat*; the remnants of the ampicillin resistance gene '*bla*; and the ColE1*ori*.

1.3 Transformation by electroporation

In this next step, attempts were made to insert the PCR-amplified integrons into the *Ecl136* II site of pTM4, introduce the ligation product in *E.coli* and obtain integron-containing plasmid DNA from the resulting transformant strain. This attempt was not successful. The initial transformation assay attempts were performed by inserting the PCR amplified fragment of the class 1 integrons into the generated plasmid pTM4 and introducing the *Ecl136*II linearised version of this plasmid (ligated to the PCR product) into *E.coli* cells by electroporation prior to the transformation assay. Despite of the successful ligation of the integrons to the flanking regions indicated by gel electrophoresis and the numerous attempts made with this method, no transformants were obtained after electroporation. The introduction of the plasmid containing the integron into the *E. coli* competent cells and subsequent transformation assays did not produce any cells that could grow on selective plates. The possible reasons for these unsuccessful attempts are discussed in Section IV. The inability to yield *E.coli* that contained the plasmid harboring the integron led to alternative attempts described in the following chapters.

1.4 Liquid transformation with PCR product

As no integron-containing vector DNA could be obtained from *E.coli*, we attempted to produce DNA segments *in vitro* which could be employed directly to transform *A. baylyi* ADP1. In the first step, the PCR amplified integron was added to a ligation reaction in which T4 DNA Ligase was used to ligate the up and down flank to the PCR amplified integron. The ligated and non ligated PCR products are shown in the agarose gel image in figure 12. The amplification of both the Ab64 and Ab65FFC integrons produced only one strong band of approximately 3000 bp where as the flanking regions where approximately 700 bp. The ligation mix between the flanking regions produced multiple bands. These multiple bands correspond to the flanking regions (approx. 700 bp and 750 bp), integron that was approximately 3000 bp and the combined product of the flanking regions and the integron which was expected to be approximately 4500 bp as shown in figure 12. Despite successful ligation of the flanking regions to the PCR amplified integron, the natural transformation assay did not yield any transformants.

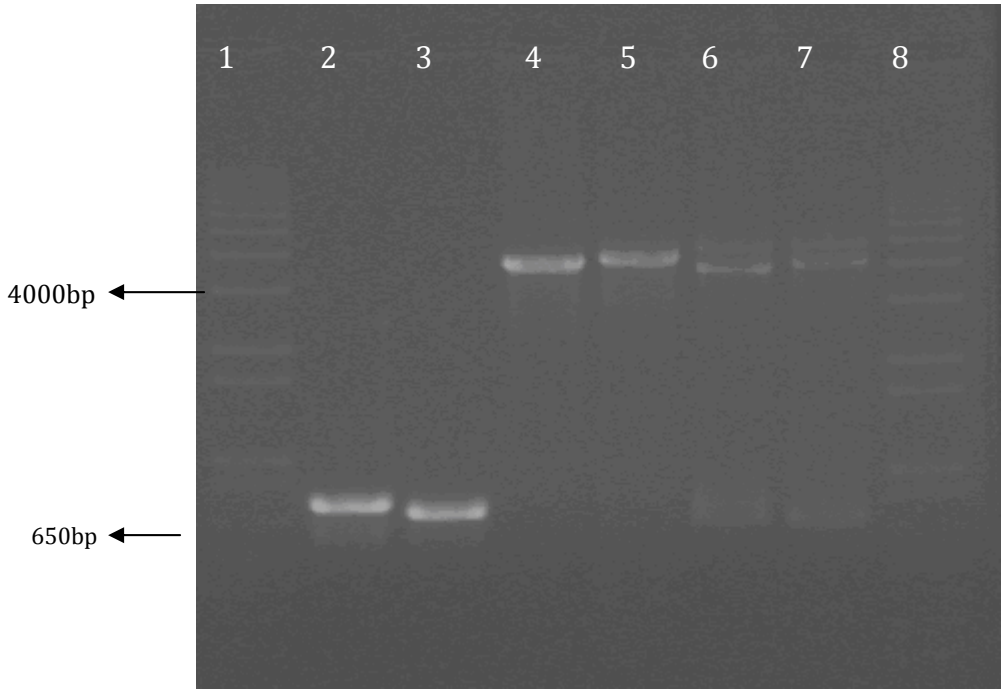


Figure 12: A gel picture illustrating the fragment sizes of the up and down flanks, the integrons in Ab64, Ab65FFC. Lane 1: Molecular weight standard 1KB+; lane 2: up flank; 3: down flank; lanes 4: PCR amplified products of integron in Ab64; 5: PCR amplified products of integron in Ab65FFC

1.5 Strain construction of *A.baylyi* with class 1 integron by splice PCR

Splice PCR employed the fusion of PCR products of the upstream and downstream flanks of ADP1 to the PCR amplified integron from strain Ab64. The procedure was conducted in steps as described in section 5.0 and *A. baylyi* ADP1 was naturally transformed with the recombinant DNA substrate. One transformant was obtained on medium containing kanamycin 50 μg/ml. This transformant Ab64.T4 was further characterized by PCR to verify the orientation of the integron in the desired locus *lifO/lipB* with primers Sul_out_F2/ downR; Sul_out_F2/ downR3 (to verify the down flank junction) and UpF/ GSrevcomp to verify the up flank junction. The constructed recombinant yielded PCR products with primers UpF/ GSrevcomp which corresponded to approximately 1650 bp hence verifying the insertion of the integron in the right position and orientation on the up flank. No PCR product however was obtained from the 3'end of the integron and the 5' end of the *lipB*. The absence of PCR product from the amplification of this region suggests that only part of the integron was inserted into the desired locus. It is possible that the second cross over in the recombination process occurred within the integron by one sided homology as is the case in illegitimate homology-facilitated recombination which only requires a random micro homology (3-12 bp) (De Vries and Wackernagel 2002). This would be a possible explanation for the transformant only containing the up flank and the newly acquired resistance to aminoglycosides in the *Acinetobacter* strain. The absence of the down flank rendered this transformant inadequate for this study therefore a different approach had to be pursued in order to obtain a transformant that possessed the desire traits.

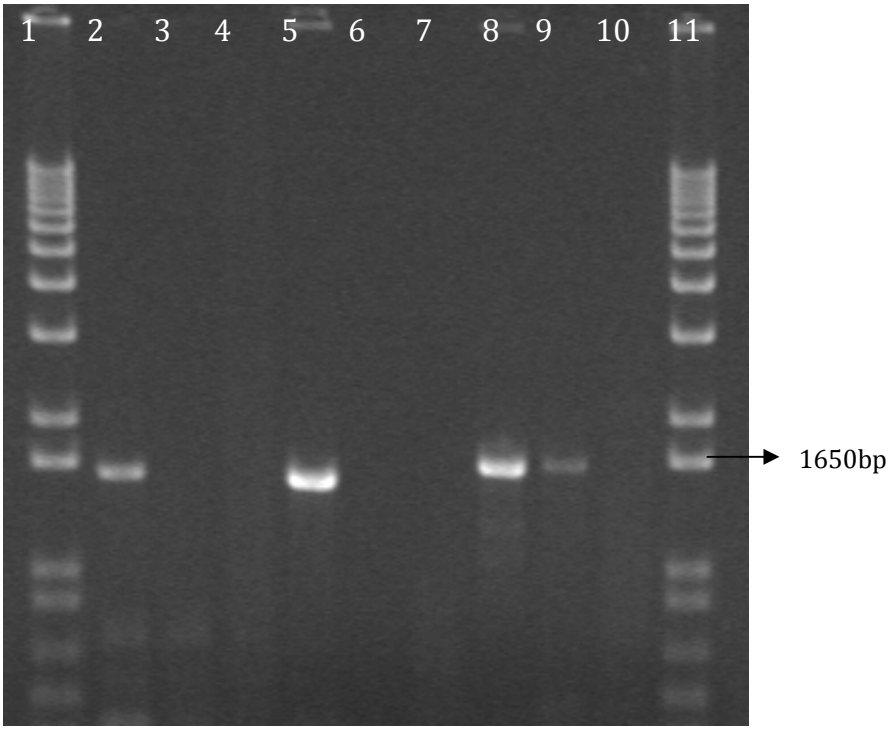


Figure 13: A gel picture illustrating the PCR products obtained from the PCR screening of recombinants obtained by splicing PCR. Lanes 1 and 11 contain the molecular weight standard 1Kb plus; lanes 2: PCR product s of Ab64.T1b amplified with Sul_out_F2/downR; lane 3 PCR product s of Ab64T4 amplified with Sul_out_F2/downR; lane 4: PCR product of ADP1 amplified with Sul_out_F2/downR. Lanes 5; Ab64.T1b amplified with Sul_out_F2/down3R; lane 6: Ab64T4 amplified with Sul_out_F2/down3R; lane 7: ADP1 amplified with Sul_out_F2/down3R; lanes 8: Ab64.T1b amplified with UpF2/GSrevcompR; lane 9: Ab64T4 amplified with UpF2/GSrevcompR and lane 10: ADP1 amplified with UpF2/GSrevcompR.

1.6 Strain construction of *A. baylyi* containing a class 1 integron by liquid transformation (without *E.coli*)

The unsuccessful transformation attempts with the methods described above required another approach. This approach was executed by ligating the plasmid pTM4 with the PCR amplified integron prior to direct application in the transformation assay as the source of donor DNA. The transformation assay was performed with the integrons in Ab64 and Ab65FFC as the donor sources and more that four transformants were obtained from each of the assays.

1.6.1 Characterization of Ab64 transformants

The transformation assay using Ab64 as the donor yielded multiple transformants however only two (Ab64.T1b and Ab64. T1d) were comparable on a genotypic and phenotypic level to the donor strain. The two transformants were screened using primer sets IntF2/orf3R; IN5'CS/ IN3'CS and aadBF/aadBR and from both transformants bands were obtained that were identical to those of the donor. Transformant Ab64.T1b was picked for subsequent characterization whilst Ab64.T1d was stored at -80°C for possible future work. The gel picture in figure 15 illustrates the results of further PCR screening conducted on Ab64.T1b. The PCR primers aadBF/aadBR were used to amplify the region containing the kanamycin resistance gene cassette (*aadB*) and gave bands that were slightly larger than 1000bp in both the donor and the transformant strains.

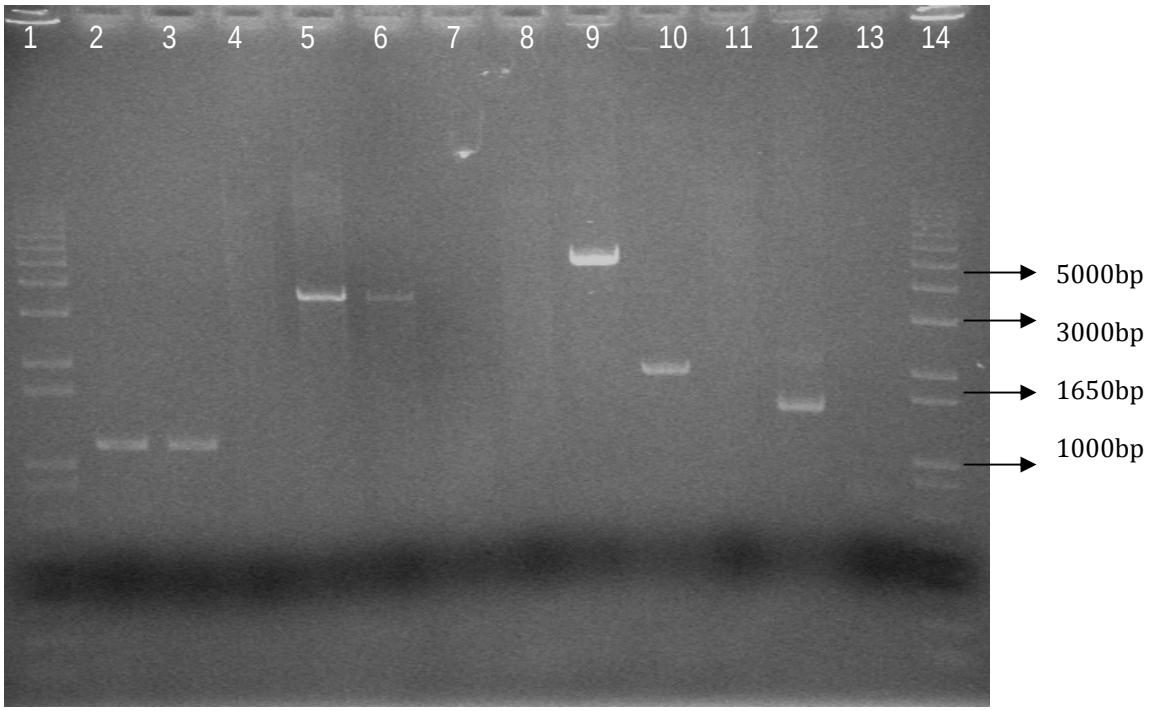


Figure 14: A gel picture illustrating the PCR products obtained from the PCR screening of Transformant Ab64.T1b. Lanes 1 and 14 contain the molecular weight standard 1Kb plus; lane 2: PCR product of Ab64 amplified with aadBF/R; lane 3: PCR product of Ab64.T1b amplified with aadBF/R; lane 4: PCR product of ADP1 amplified with aadBF/R; lane 5: Ab64 amplified with IntF2/Orf3R; lane 6: Ab64.T1b amplified with IntF2/Orf3R; lane 7: ADP1 amplified with IntF2/Orf3R; lanes 8: Ab64 amplified with upF/downR; lane 9: Ab64.T1b amplified with upF/downR; lane 10: ADP1 amplified with upF/downR. Lane 11: Ab64 amplified with primers sulF2/downR; lane 12: Ab64.T1b amplified with primers sulF2/downR and lane 13: ADP1 amplified with primers sulF2/downR.

The integrons in both the Ab64 and the Ab64.T1b which were found to be approximately 4000bp were amplified with IntF2 and Orf3R whilst primers UpF2 and downR which amplified the *lifO/lipB* region produced no bands in the donor but a band that was approximately 6000 bp in the transformant and only 2000 bp in the recipient. The absence of bands in the donor strain was expected as the strain is not known to contain this region and the 6000 bp band that was observed in the transformant Ab64.T1b indicates the insertion on the approximately 4000 bp integron segment in the region between *lifO* and *lipB*.

The PCR primers (Sul_F2/downR) that amplify a region between the *sul*1 gene and the down flank (*lipB*) only yielded a band in the Ab64.T1b which was approximately 1650 bp. The recipient ADP1 which was used as the negative control for the gene cassette, integron and the flanking region joined to the integron did not yield any bands with primers aadBF/aadBR, IntF2/Orf3R and Sul_F2/downR respectively. It was however used as a positive control with primers UpF2 and downR where the insertion of the integron resulted in a 4000 bp increment.

The nucleotide sequencing of PCR products obtained from this strain showed that the integrase gene in this strain was 99% identical to that found in *A. baumannii* strain AB08-ColR (Accession number GQ281659.1). The generated sequence of the *aadB* gene showed 96% homology with *A. baumannii* strain carrying a class 1 integron (Accession number AJ89190). The junction between the 3' end of the integron and the 3' end of the lifO region was sequenced and the sequence in the obtained from the transformant was found to have 99% similarity to the *lifO* gene in ADP1 over a length of 629 bp (Barbe et al. 2004) and 96% similar to the sul1/Orf5 of *A. baumannii* 3208 isolated from Australia which is a multiple resistance strain containing class 1 integron (Post and Hall 2009). Based on the sequence verification of this new strain of *A. baylyi* carrying class integron, the strain was used in the study to investigate the stability and the fitness cost of a newly acquired class 1.

1.6.2 Screening of Ab 65FFC Transformants

The transformants obtained from Ab65FFC assay (Ab65.T1-T12) possessed the expected phenotype (resistance to ampicillin). The agarose gel image in figure 14 illustrates the results of the genotypic tests by PCR (with primers that amplify the *bla-*$_{IMP2}$ gene cassette and the conserved segment of class 1 integrons) which revealed differences between the donor strains and the transformants. Transformants T1, T2, T3, T9 and T10 when amplified with primers for the *bla-imp* gene cassettes did not produce any PCR product whilst transformants T4, T5, T6, T7, T8, T11 and T12 produced PCR produced that were visualized as single bands. However when these bands were compared to those of the donor strains PCR product from the same primers, the transformant PCR products differed in size by approximately 150 bp. The donor strain produced a band that was approximately 500 bp whilst the named transformants were approximately 650 bp. When amplified with the integron primers IntF2 and Orf3R, the donor gave band two bands that were 3000 bp and 1650 bp. On the other hand the transformants T5, T6, T7 and T11 produced a single band that was equivalent to 1000 bp. Transformant T4 produced two bands of approximately 2000 bp and 1000 bp and transformants T8 and T12 did not show any bands. The PCR products (obtained from the conserved region primers) of the transformants were sequenced however the sequences contained too many unspecified nucleotides to allow adequate comparison with the donor strain. The poor sequencing result may have been due to high DNA concentration in the sequencing reactions or inadequate purification of PCR product prior to the sequencing reaction.

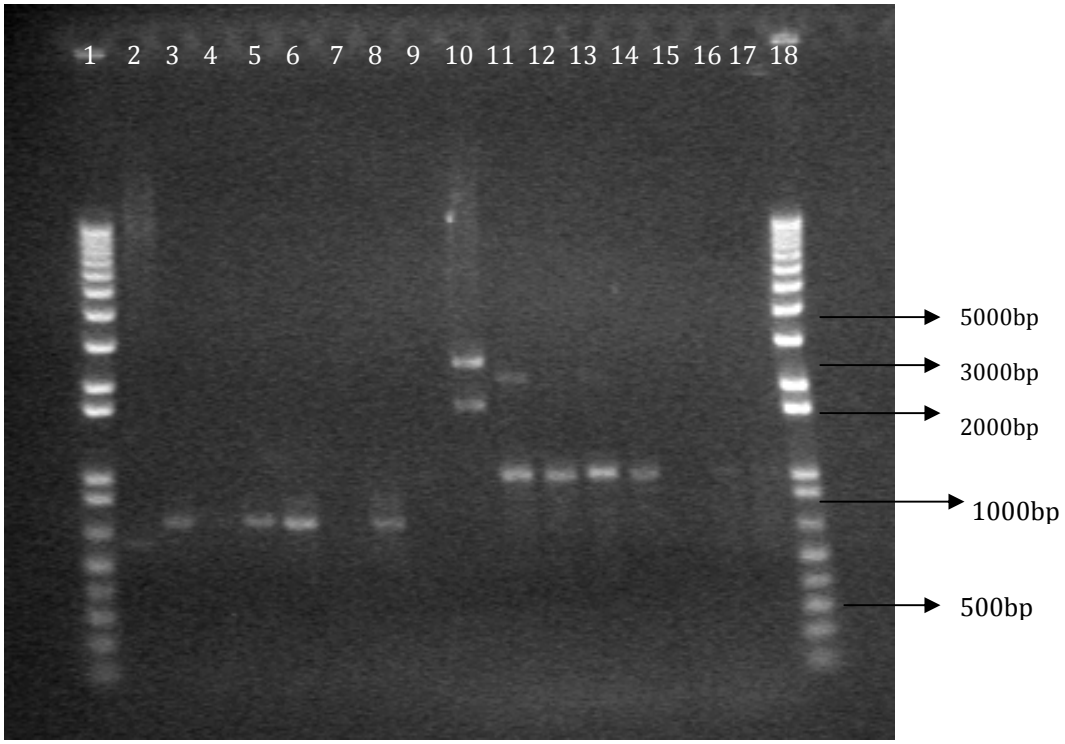


Figure 15: A gel picture showing the PCR products visualized on 1% agarose gel that were amplified from Ab65FFC transformants (T4, T5, T6, T7, T8, T11 and T12) with primers the amplify the ampicillin resistance gene cassette, (bla_{IMP5}) and the harboring integron. Lane 1 and 18 contain the molecular weight standard 1Kb plus; lane 2: Ab65FFC amplified with primer impL/IimpU; lane 3: T4; lane 4: T5; lane 5: T6; lane 6: T7; lane 7: T8; lane 8: T11; lane 9: T12. The samples in lanes 10 to 17 were amplified with the conserved primers IN5'CS and IN3'CS with lane 10: T4; lane 11: T5; lane 12: T6; lane 13: T7; lane 14: T8; lane 15: T11; lane 16: T12 and lane 17 was loaded with ADP1.

1.6.3 Construction of strain Ab64.T1b *int::cat*

The previously constructed stain Ab64.T1b was designed to determine the biological cost of the integron on the cells. To determine the cost of the integrase gene on the bacteria only, another strain (Ab64.T1b *int::cat*) was constructed and this strain varied from strain Ab64.T1b in that it carried the class 1 integron with an inactive integrase gene. This strain was obtained by natural transformation of strain Ab64.T1b by linearised pACYC177-int-*cat* DNA. This vector contained a 5'- and 3'- truncated internal fragments of the *int1* gene of strain Ab64 with a chloramphenicol resistance gene inserted roughly in the center of that segment. Transformants were obtained on medium containing 10 µg/ml.

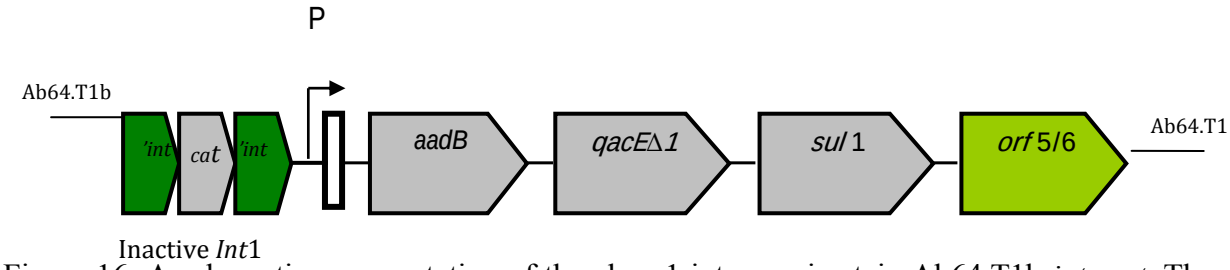


Figure 16: A schematic representation of the class 1 integron in stain Ab64.T1b *int::cat*. The figure illustrates the position of the chloramphenicol resistance gene (*cat*) within the integrase gene *int;* P: the promoter; *aadB*: aminoglycoside resistance gene; *qacEΔ1*: quaternary ammonium resistance gene; *sul1*, sulphonamide resistance; *orf5*, a gene of unknown function; within the genome of Ab64.T1b.

The transformants were screened by PCR with primer IntF2 and orf3R in order to verify the size increment due to the presence of the chloramphenicol gene in the integrase. As shown in the image below (figure 17) the integrons in strains Ab64 and Ab64.T1b are approximately 4000 bp where as the one present in strain Ab64.T1b *int::cat* is approximately 5000 bp.

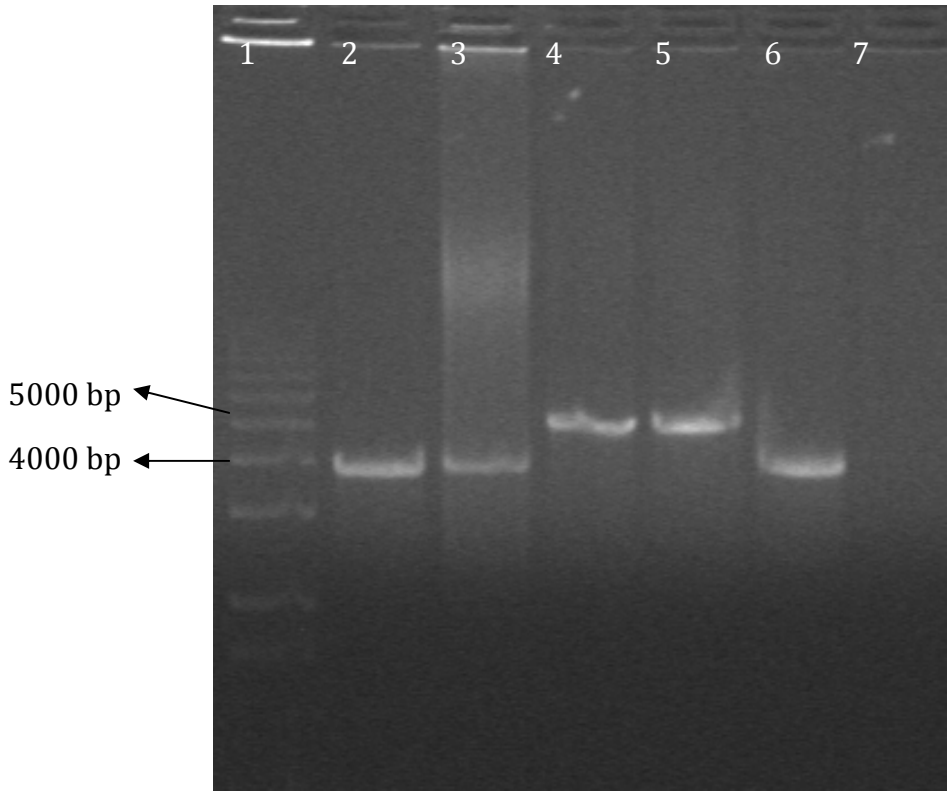


Figure 17: An agarose gel image of PCR products amplified with IntF2 and Orf3R. In lane1: molecular weight marker 1Kb plus, lanes 2 and 3: Ab64.T1b; lanes 4 and 5: Ab65.T1b *int::cat*; lane 6: Ab64 as positive control and lane 7: ADP1 as the negative control. The image shows the increase in size of the integron in strain Ab64.T1b *int::cat* due to the insertion of the chloramphenicol gene.

1.6.4 Strain construct of *A. baylyi* with *nptII* and *sacB* gene cassettes

The neutrality of the insertion locus *lifO/lipB* was tested with strain ADP1.*nptII.sacB* which is an *Acinetobacter* strain carrying the *nptII* and *sacB* cassettes are selectively neutral. This strain was designed to verify that neither the cassettes nor the insertion site conferred any cost to the host strain; it was obtained by transformation of *A. baylyi* ADP1 by linearised pTM2 DNA. The transformation assay yielded transformants that were screened based on sensitivity to sucrose which was imparted by the *sacB* gene and kanamycin resistance which was inferred by the *nptII* gene. The screening of the transformants screen for the correct insertions was conducted by Irina Starikova using the primer set UpF/DownR and UpF3/down3R and the visualized PCR products are shown in figure 18 below. Only one of the transformants, T1 shown in lanes 2 and 13 produced PCR that correspond to approximately 4000 bp. The negative control ADP1 and Ab64.T1b when amplified with primer UpF and downR produced PCR products of 2000 bp and 5000 bp respectively which corresponded to the expected PRC product sizes. The arrangement of the gene cassettes inserted in ADP1 is illustrated by figure 18.

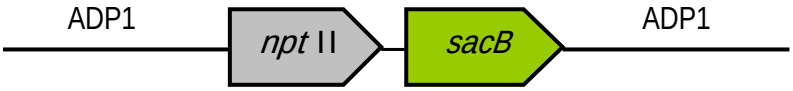


Figure 18: A schematic representation of the *nptII* and *sacB* cassettes inserted into the model organism ADP1. The *nptII* gene confers resistance to kanamycin where as the *sacB* gene cassettes confer sensitivity to sucrose.

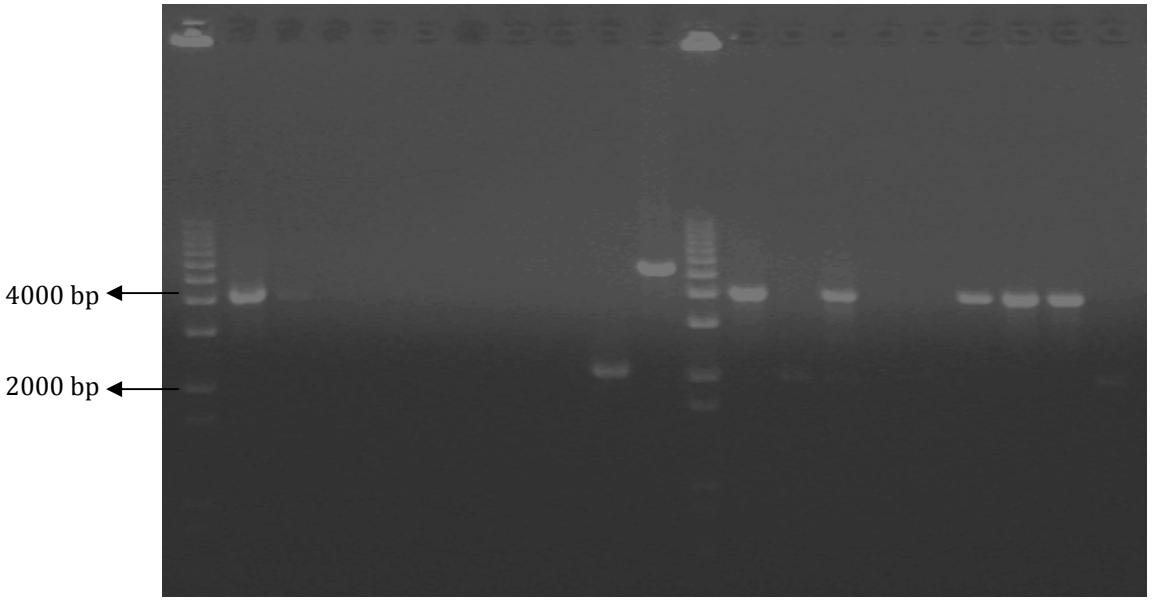


Figure 19: An image of 1% agarose gel loaded with PCR products used in the screening of transformants from the construction of the ADP1.*nptII.sacB*. Lanes 1 and 12 contain molecular mass marker, 1Kb plus; lane 2: transformant 1; lane 3: transformant 2; lane 4: transformant 3; lane 5: transformant 4; lane 6: transformant 5; lane 7: transformant 6; lane 8: transformant 7; lane 9: transformant 8; lane 10: ADP1; lane 11: Ab64.T1b – all amplified with primers UpF/downR. The primers UpF3 and downR3 where used to screen for transformants and in lane 13: transformant 1; lane 14: transformant 2; lane 15: transformant 3;lane 16: transformant 4; lane 17: transformant 5; lane 18: transformant 6; lane 19: transformant 7; lane 20: transformant 8; lane 21: ADP1; lane 22: Ab64.T1b.

2.0 Gene cassette recruitment

The addition of new gene cassettes to the newly constructed strain AB64.T1b was performed using the plasmid free liquid transformation method as described in section 5.8. The strains Ab65FFC, K34-74 and A3-54 were used as sources of additional gene cassettes which conferred resistance to ampicillin and meropenem. The transformation assay that used Ab65FFC as a source of new gene cassettes yielded five transformants. However upon further analysis these transformants proved not to contain the desired gene cassettes. The strain A3-54 did not produce any transformants whilst strain K34-74b yielded four transformants of which Ab64.T1b.Da and Ab64.T1b.Db. These transformants were phenotypically tested and were found to be resistant to kanamycin, ampicillin and meropenem (which the initial recipient was susceptible to). They were grown on plates that contained a combination of ampicillin 100 µg/ml and meropenem 25 µg/ml and /or Kanamycin 50 µg/ml and meropenem 25 µg/ml. The two transformants were screened with primers IntF2/qac which are primers inwards on the integron and these yielded a 2000 bp product in both and also in the positive control which was the donor strain AB64. The negative control ADP1 did not produce any visible PCR product with these primers. The attachment of the integron to the desired up flank

(*lifO*) was confirmed genotypically with primer sets UpF/GSRevcomp which amplify the region between the *lipB* gene and the integrase. This PCR produced single bands in each sample which correspond to 1650 bp. The donor strain which was used as a negative control did not produce any bands where as the recipient strain Ab64.T1b which initially contained this region yielded a strong single band.

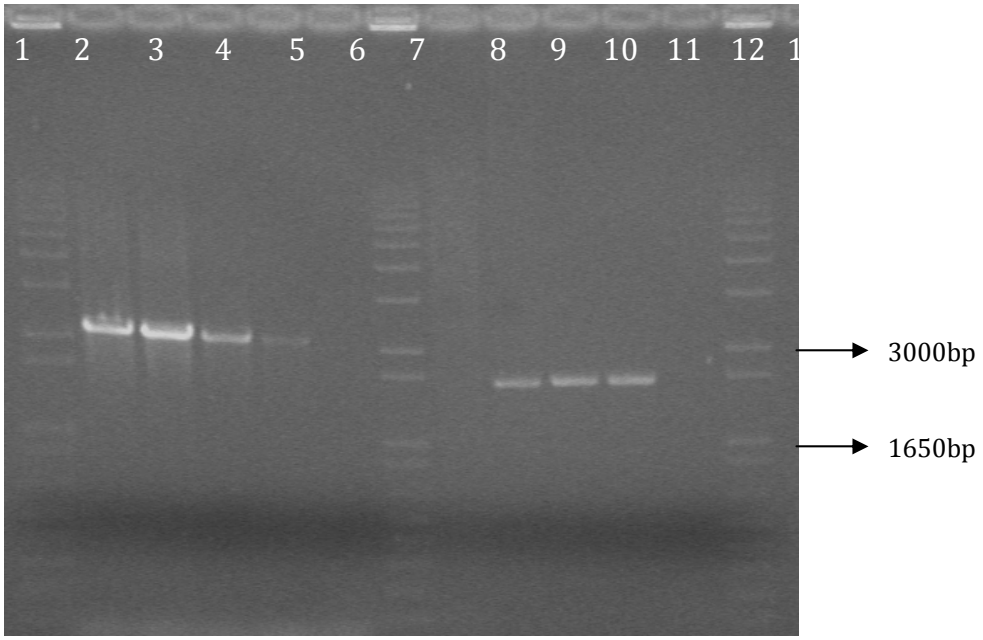


Figure 20: An image of a 1% agarose gel loaded with samples from the recruitment of gene cassettes into the newly acquired integron Ab64.T1b. Lanes 1, 7 and 13: Molecular weight standard 1Kbp plus; lanes 2: Ab64 amplified with primers IntF2 and qac; lane 3: Ab64T.1b amplified with primers IntF2 and qac; lane 4: Ab64.T1b.Da amplified with primers IntF2 and qac; lane 5: AB64.T1b.Db amplified with primers IntF2 and qac and lane 6: A3-53 amplified with primers IntF2 and qac. Lanes 8: Ab64 amplified with primers UpF and GSrevcomp; lane 9: Ab64T.1b amplified with primers UpF and GSrevcomp; lane 10: Ab64.T1b.Da amplified with primers UpF and GSrevcomp; lane 11: AB64.T1b.Db amplified with primers UpF and GSrevcomp and lane 12: A3-53 amplified with primers UpF and GSrevcomp.

3.0 Susceptibility and Resistance tests

3.1.1 Disc susceptibility test

The susceptibility patterns of donor, recipient and transformants were determined using the Disc diffusion method which is based on the diffusion of antibiotics through a growth medium that is covered by a lawn of bacterial cells. The susceptibility patterns of all the strains is given the in the table below. The recipient strain ADP1 was found to have the highest level of susceptibility to all the antimicrobial agents used during this study with the largest inhibition zone being 40 mm with 10 µg/ml imipenem. The donor strain Ab64 displayed resistance to chloramphenicol, sulphonamide and the aminoglycosides (gentamicin,

kanamycin and streptomycin). The strain was previously reported as containing the *aadB* gene (Da Silva, unpublished) which encodes for aminoglycoside adenylyltransferase, an enzyme that bind to the bacterial ribosome and inhibit protein synthesis (Vakulenko and Mobashery 2003).

Table 6: Table giving the inhibition zone from the antibiotic susceptibility tests of the test strains

Strain	**CAZ**	**CL**	**CTX**	**GM**	**IMP**	**KM**	**SUL**	**SH**
Ab64	20	0	12	0	37	0	0	0
Ab65FFC	0	0	0	25	0	27	0	20
Ab64.T1b	20	0	12	25	36	10	0	20
Ab64.T1b.Da	0	20	0	0	10	0	0	20
ADP1	20	20	25	27	40	30	30	20
K34-74 (St.D)	Nd	0	Nd	Nd	0	Nd	Nd	Nd
A3-54 (St.E)	0	0	Nd	0	Nd	Nd	Nd	Nd

CAZ- Ceftazidime 30 µg/ml; CL- Chloramphenicol 30 µg/ml; CTX- cefotaxime 30µg/ml; GM –Gentamicin 10 µg/ml; IMP- Imipenem 10 µg/ml; KM- Kanamycin 30 µg/ml; SUL- Sulphamonide 0.25 µg/ml; SH- Streptinomycin 25 µg/ml, Nd- Not done

The transformant Ab64.T1b displayed similar resistance and susceptibility patterns to the donor with the exception of susceptibility to kanamycin, streptinomycin and gentamicin which were illustrated by inhibitory zones of 10 mm, 20 mm and 25 mm, respectively. On the other hand, strain Ab65FFC was only susceptible to the aminoglycosides and showed resistance to the cephalosporins (ceftazidime and cefotaxime), chloramphenicol and imipenem. The strain harbors the *bla-$_{IMP-5}$* which confers resistance to broad spectrum cephalosporins and carbopenems therefore the obtained results were expected. The *P. aeruginosa* strains K34- 74 and A3-54 displayed resistance to the beta lactams, cephalosporins and the aminoglycosides used in this study and this is an accordance with previously obtained results (Samuelsen et al. 2009) .

3.1.2 Minimal Inhibitory Concentration by E-test

The minimal inhibitory concentration (MIC) of the donor, recipient and transformants was determined using the E-test as described in section 7.2 and are listed in table 7 below. All strains with the exception of the recipient strain ADP1, displayed patterns of multi drug resistance with the highest levels of resistance being observed in the *P. aeruginosa* strains were found to have MICs above 256 µg/ml for all the antibiotics used in the study with the exception of strain K34-74 which displayed MICs of 96ug/ml and 48 µg/ml to rifampicin and streptomycin respectively. The multiple resistance patterns displayed by the *P. aeruginosa* strains are similar to those observed by (Samuelsen et al. 2009) and are attributed to the presence of multiple gene cassettes on the integrons that these strains contain. Strains Ab 64 and Ab65FFC were found to be resistant to cefotaxime, gentamicin, kanamycin, and rifampicin with the highest resistance corresponding to kanamycin for Ab64 and cefotaxime and Rifampicin in Ab65FFC. The resistance levels in the transformant Ab64T1.b were 4 µg/ml, 4 µg/ml, 24 µg/ml and 3 µg/ml to cefotaxime, gentamicin, kanamycin and rifampicin respectively whilst transformant Ab64.T1b.Da was found to have resistance above 256 µg/ml to ceftazidime, cefotaxime and kanamycin and 2 µg/ml and 1.5 µg/ml to rifampicin and streptomycin respectively. The recipient strain ADP1 illustrated resistance levels of 4 µg/ml, 0.25 µg/ml, 1 µg/ml and 3 µg/ml to cefotaxime, gentamicin, kanamycin and rifampicin respectively. When compared to the recipient, it is observed that the transformant has higher resistance levels to gentamicin and kanamycin (from 4 µg/ml and 1 µg/ml to 4 µg/ml and 24 µg/ml respectively). In strain Ab65FFC, the resistance to cefotaxime and rifampicin was above the detection levels of 256 µg/ml whilst the 0.5 µg/ml to gentamicin and 2 µg/ml to kanamycin.

According to the interpretive criteria provided by AB BIODISK, Sweden, strain Ab64.T1b is susceptible to ceftazidime, intermediately resistant to cefotaxime and resistant to kanamycin and gentamicin where as the MIC values from the recipient strain ADP1 would have it classified as susceptible to all the afore mention antimicrobial agents. The donor strain AB64 would be classified as resistant to kanamycin and gentamicin, intermediately resistant to cefotaxime and susceptible to ceftazidime. In spite of harboring the entire integron from the donor strain, the transformant expressed lower resistance to all the antibiotics included in the test and shows different patterns when interpreted. This may be attributed to various reasons

with the most likely being differences in promoter activity leading to lower expression in the transformant.

Table 7: Table illustrating the Minimal inhibitory concentration of the test strains.

Strain	**CAZ** µg/ml	**CTX** µg/ml	**GM** µg/ml	**KM** µg/ml	**RI** µg/ml	**SH** µg/ml
Ab64	6	50	48	>256	8	96
Ab65FFC	>256	>256	0.5	2	>256	*Nd
Ab64.T1b	4	4	4	24	3	4
Ab64.T1b.Da	>256	>256	*Nd	>256	2	1.5
ADP1	4	4	0.25	1	3	4
K34-74 (St.D)	>256	>256	>256	>256	96	48
A3-54 (St.E)	>256	>256	>256	>256	>256	>256

CAZ- Ceftazidime ; CTX- cefotaxime; GM –gentamicin; KM- Kanamycin ; SH- Streptinomycin

*Nd- Not done

Table 8: Table of antimicrobial agents and interpretive criteria adopted from AB BIODISK, Sweden.

Antimicrobial agent	**Susceptible ≤ µg/ml**	**Intermediate µg/ml**	**Resistant ≥ µg/ml**
Ceftazidime	8	16	32
cefotaxime	8	16-32	64
gentamicin	4	8	16
Kanamycin	16	32	64

4.0 Fitness Competitions

The fitness cost of harboring a newly acquired integron was assessed by a pair wise competitions studies between the model organism and each transformant over a period of 24hrs. The fitness cost of each experiment was calculated using the Malthusian parameter as described in section 8.0 of the materials and methods. The fitness competitions involving strains Ab64.T1b *int::cat* and ADP1.*nptII.sacB* were conducted by Irina Starikova. The results from all three studies were pooled and show that in strain Ab64.T1b, the relative fitness of harboring an integron was found to be 0.93 (± 0.01 Standard deviation) which translates to an average fitness cost of 7%. The fitness cost of carrying an integron with an inactive integrase gene was investigated with strain Ab64.T1b *int::cat* which carries the integrase gene inactivated by the insertion of a chloramphenicol gene (cat). The relative cost of the inactive integrase containing strain was found to be 0.98 (± 0.02 S.D), which translates to a fitness cost of 2%, and this value is significantly different from zero. As a control, the selectively neutral cassettes *nptII* and *sacB* were inserted in the selective neutral locus of *lipA/B* and in this case the relative fitness was found to be 1 which corresponds to 0% fitness cost (no difference in relative fitness). A graphical representation of the results is given in figure 19.

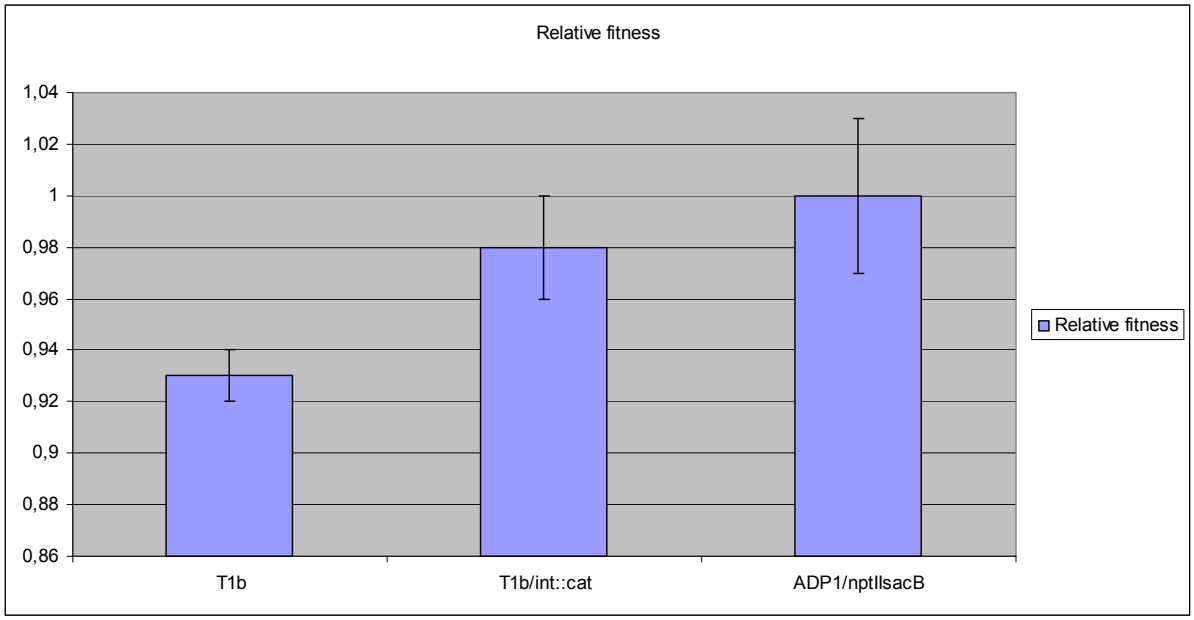


Figure 21: A graphically representation of the relative fitness cost of the integron in strain Ab64.T1b; integron with an inactive integrase in strain AB64.T1b *int::cat*; and ADP1 carrying selectively neutral cassettes *nptII* and *sacB*.

5.0 Stability test

The stability of the gene cassettes in the class 1 integrons found in the strains Ab64, Ab64.T1b, K34-74 and A3-58 that were used in this study was accessed by replica plating over a period of 10 days as described in section 9.0. The strains Ab64 and Ab64.T1b were grown on LB plates containing kanamycin (50 µg/ml), sulphonamides (20 µg/ml) and LB plates without selection. The *P. aeruginosa* strains (K34-74 and A3-58) were grown on kanamycin (50 µg/ml), and/or ampicillin (100 µg/ml) and LB plates without any antibiotics. All four strains where able to grow in the presence of the selective pressure thus indicating that they maintained the resistance to the selected antibiotics. This demonstrates that the integrons in the test strains were 100% stable with less than 1 in 1000 exhibiting cassette loss in the absence of selection over the ten day period. The initial stability studies were conducted in triplicate with no selective pressure added however as the integrons seemed stable, the studies were repeated in triplicate with two parallels in which one included selective pressure and the other was not subjected to selective pressure. The parallel studies did not produce any variations in the stability of the integron. The screening for loss of resistance after ten days showed that less than 1 in 1000 colonies lost the resistance phenotype in the presence or absence of selection. The graph below given in figure 20 is a representation of only one of the triplicate studies in the parallel studies.

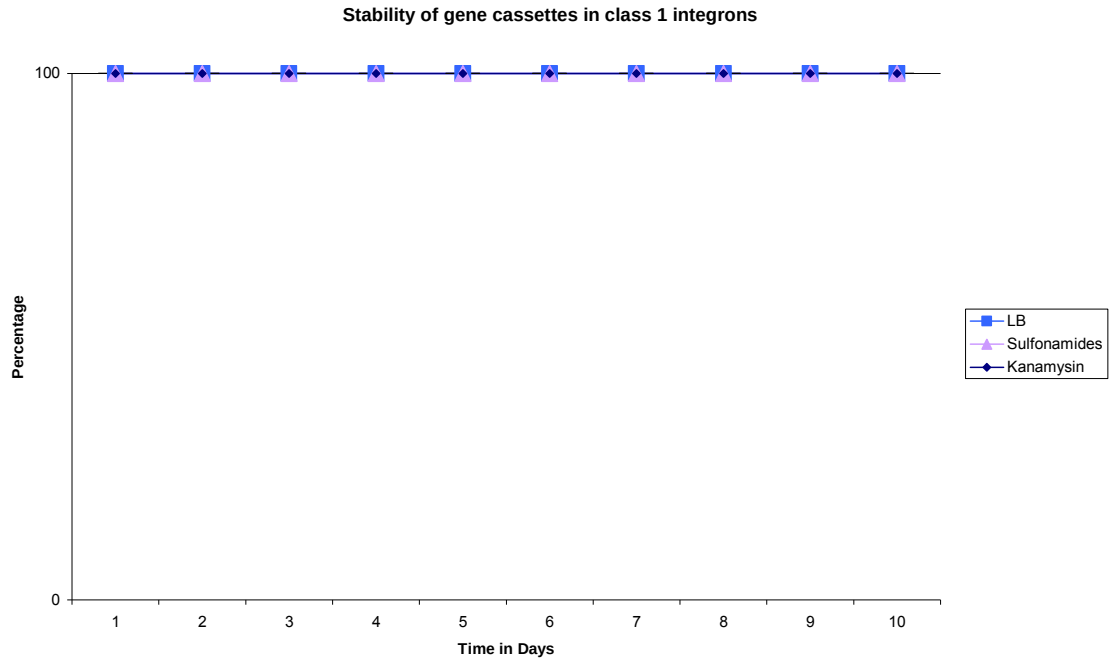


Figure 22: A graph illustrating the stability of the integron and harbored gene cassettes over a period of 10 days.

5.1 Cassette Excision

Gene cassettes are known to exist in a circular form when not integrated in an integron or when excised from the integron. With this in mind, the stability of the gene cassettes in the newly acquired integrons in strain Ab64T.1b was further investigated by cultivating the strain in an antibiotic free environment and then extracting all genomic DNA from the cells which was screened for excised cassettes. The primer sets aadBForRight1/ aadBleftR1, aadBForRight3/ aadBleftR3, aadBForRight1/aadBleftR2 and aadBForRight3/ aadBleftR2 were used in the search for excised and only latter two primer sets (which were used as positive controls) produced PCR products of 500 bp and 300 bp respectively. The position of the primers that produced the bands does not require for the cassette to be in a circular form as they positions allow the production of PRC product in the integrated cassettes. Based on addition consultation Irina Starikova has since run other PCR reactions where the PCR product from this study was used as template. These reactions have yielded bands indicating the presence of re- circularized gene cassette but as further work is required to verification, these finding are not included in the report.

SECTION IV: DISCUSSION

1.0 The biological cost of newly acquired class 1 integrons

The increase of antimicrobial resistance among bacterial populations raises the need to understand factors that contribute to this increase and promote the maintenance of resistance in bacterial populations. The biological cost that is associated with the acquisition of a new trait is considered as one of the main parameters that determine whether or not the trait will be maintained and/or spread in the bacterial population in the absence of direct selection. This study assessed the fitness effects of carrying an integron by conducting the first ever estimates of the biological cost of a newly acquired class 1 integron. ADP1, a naturally transformable, prototrophic bacterium that has simple culture requirement was used as the host strain for a class 1 integron of clinical origin. The significance of using ADP1 as the recipient strain in this study lies in that;

(i) ADP1 is not known to contain any integrons as determined by whole genome sequencing (Barbe et al. 2004) or to have had any known association or contact with integrons.

(ii) ADP1 is closely related to the more problematic *A.baumannii* which is an opportunistic human pathogen that has been known to cause serious infections in humans, especially in immuno-compromised patients (Tomaras et al. 2008). The growing number of community and nosocomial infections caused by *A. baumannii* coupled with the emergence of multi resistant strains raises the need to understand this microorganism better. However in comparison to *A. baylyi*, *A. baumannii* has not be known to be easily transformable (Chen et al. 2008). A recent paper suggests that at least one *A. baumannii* strain may be transformable but it has a multiple resistant phenotype that hinder detailed molecular studied (Ramirez et al. 2010).

The acquisition of a new trait requires that the bacteria replicate the additional DNA and synthesize novel proteins thereby increase the burden on the organism. In cases where the acquired trait is antimicrobial resistance and the bacteria is growing in the presence of selective pressure, the bacteria bears the extra burden of maintaining the resistance trait as this will enable the organism to survive. However in the absence of selective pressure, the bacteria will be maintaining a trait that confers a biological cost without any benefits thus it is

expected that the resistant determinants will be selected against and the levels of resistance will decrease in the population (Low et al. 1999). In this study, a class 1 integron was introduced into *A. baylyi* and the biological cost was assessed by relative fitness studies. The fitness cost of the newly acquired class 1 integron on the host strain was found to be 7% in pair wise competition that were performed between the transformant Ab64.T1b and the otherwise isogenic model organism ADP1. This cost is considered very high especially when compared to other studies that have given similar fitness costs. One case in point is the fitness cost of 7% that resulted from mutations in the 16S rRNA gene at position C1192G that was observed in *Chlamydia psittaci* (Binet and Maurelli 2005). Mutations in the chromosomal such as those in the 16S rRNA affect essential functions in bacterial physiology thus it is expected that the burden placed on the bacteria is large. In the case of this study where the class 1 integron was inserted into a selective neutral locus, the observed high fitness cost was assumed to be caused by the integron and is independent of the insertion site in the genome. The next natural question is what the practical consequences of a 7% reduction in relative fitness are. Levin and colleagues used a mathematical model on the population genetics of antibiotic resistance and under arbitrary but somewhat realistic parameter values they showed that it would take approximately two years for a bacterial population to reduce the frequency of resistance from 50% to the level where resistance was regenerated by HGT or recurring mutations (Levin et al. 1997). Two years may seem as a short period in terms of evolution but this mathematical theory is based on the assumption that there is a complete termination of use of antimicrobial selection without which resistance level would drastically increase.

The observed fitness cost coupled with the initial problems faced during the transformation by electroporation assays raised the interesting question of the role that the integrase played in this process. Previous studies by Airenne and others ad shown that a member of the tyrosine recombinase family (Cre) had a toxic effect on living cells (Airenne et al. 2005). Thus, it could be hypothesized that the integrase (also a member of the tyrosine recombinase family) could play a role in imposing a biological cost on the host strain. This hypothesis was tested by carrying out fitness cost studies with an integrase knock out strain Ab64.T1b *int::cat*. The integrase in the strain was inactivated by the insertion of a chloramphenicol gene therefore any cost that would be impaired on the strain would not be expected to come from the integrase but from the rest of the integron. This strain was subjected to pair wise competition against the model organism ADP1 and it was found that the integron carrying the inactive integrase impose a fitness cost of 2%. When compared to the strain carrying an active

integrase gene, it becomes evident that the bacterial strain bears a cost 5% by carrying an integrase gene. These results clearly suggest that the integrase is a biologically expensive genetic element maintain as it confers a high fitness cost. As the fitness cost of a class 1 integron with an active integrase was high, it was necessary to verify the neutrality of the insertion site in *A .baylyi*. This was achieved by inserting selectively neutral cassettes (*nptI*I and *sacB*) into the exact same locus (*lifO/lipB*) as the integron was inserted. Competition studies between stain ADP1.*nptII.sacB* and the model organism revealed that the cassettes did not impose any fitness cost on the host strain in support of P. Johnsen unpublished results. The high fitness cost associated to harboring class 1 integron that are demonstrated in this study go against the hypothesis which stated that the widespread dispersal of integrons in gram negative bacteria is due to low if any biological cost associated with harboring class 1 integrons.

Integrons have been described as genetic elements that by means of natural cloning and an expression system can capture, incorporate and transform gene cassettes into functional genes (Rowe-Magnus and Mazel 2001). Integrons are ubiquitous in nature and have been found in a wide range of environmental bacterial species taken from markedly different sources (Mazel 2006). The widespread dispersal of integrons in nature, the unique ability of an integron to capture, integrate and express genes that are on a gene cassette and their association with antibiotic resistance genes which has rendered them an important factor in the increasing levels of multi- drug resistance (San Martin et al. 2008) would lead to the speculation that maintenance of these genetic elements does not have a high fitness cost in bacterial populations. This is contrary to the findings in this study. The presented data show that our constructed integrase insertion-deletion clearly compensated for the costs. The results presented in this thesis may very well explain why non-functional integrases are common (Nemergut et al. 2008). If, and how bacteria may further adapt to the biological cost of carrying class 1 integrons is an ongoing topic for research in our group. Our experimental design, with the use of a strain that has possibly never been in contact with integrons is optimized for revealing fitness effects of integron carriage. It would also allow us to further study alternatives to integrase knockouts/deletions as compensatory mechanisms. This could be done by allowing Ab64.T1b to evolve in the laboratory and characterize high fitness mutants.

With fitness levels as high as 7% it would be expected that in the absence of selection pressure, the bacteria would attempt to reduce the burden of harboring genetic elements that

did not provide any beneficial traits as stated by Low and others (Low et al. 1999). It is possible that that the remaining 2% of reduced fitness could be related to the expression of the cassette. It could be expected that the bacteria would either lose the entire integron or excise the cassettes and revert to susceptibility when cultivated in an environment that is free of selective pressure. The ability of the integrons to retain the resistance genes in the absence of antimicrobial presence was found to be 100% as less than one in a thousand colonies lost the resistance phenotype when cultivated in the absence and /or presence of antibiotics over a period of 10 days which corresponds to approximately 70 generations. The observed stability may be attributed to several factors among which insufficient experimental time is the most likely. Based on the model for population genetic of antibiotic resistance by Levin and others from 1997 (Levin et al. 1997) a fitness cost of 7% (for the entire integron) and 2% would approximately take 200 or more than 500 generations (loss of a single cassette) before the loss of resistance could be detected. Further studies are currently underway in which the length of the experiment has been extended to cover the theoretical number of generations needed to observe excision of cassettes.

The loss of integrons from a bacterial population in the absence of selective pressure has been reported in the literature and the presented data suggested that the removal of selective pressure would result in integron loss (Norrby 2005). The mechanism involved in the loss of the integron is however not clearly stated and based on an earlier study by Rosser and Young it is tempting to speculate that the loss of integrons actually refers to the loss of the gene cassettes in the integron (Rosser and Young 1999). In nature, integrons are found in association with insertion sequences and composite transposons that are present on transposons and plasmids thereby provided the integron with a mode of movement (Boucher et al. 2007) therefore the removal or loss of the integron from strain Ab64.T1b would expectedly require the aid of an external genetic element such as transposons or a plasmids. As interesting as this idea seems, it lies outside the scope of this study but serves as an interesting topic for future studies.

A study by Guerin and others suggests that the stability and acquisition of gene cassettes in an integron are controlled by an SOS response system. In this study a conserved LexA-binding motif overlapping the putative promoter regions in several integrase genes of chromosomal integrons and mobile integrons (Guerin et al. 2009). The LexA protein controls the expression of many of the genes in the SOS regulon and it acts as a transcriptional repressor of these unlinked genes by binding to specific sequences (LexA boxes) located within the promoter

region of each LexA-regulated gene (Fernández et al. 2000). The Guerin study illustrated that upon SOS induction, cassette excision rates in class 1 mobile integrons and *V. cholerae* chromosomal integrons increased by 141-fold and 340-fold, respectively thereby demonstrating that the regulation of the integrase genes are strictly dependent on the SOS response and that SOS induction controls the rates of cassette recombination. The study also reported that under normal conditions, the SOS repression of *intI* maintains integron cassette arrays in a steady state whereas under stressful conditions the SOS response that led to gene cassette excision. Based on these findings the gene cassettes in an integron would be expected to be stably maintained until or unless selective pressure (such as antibiotic induced stress) is applied. Theses findings may be a partial explanation for the stability of gene cassettes observed in this study however as the studies were conducted in both selective and non selective environment this only applies to part of the study. If indeed the induction of the SOS leads to gene cassette excision, a prolonged study on the stability of gene cassettes in a newly acquired class 1 integrons in the presence and absence of stress would be an interesting area for further research.

Compensatory mutations are genetic alterations that occur within the bacterial population in order to reduce the host-fitness cost of the resistance trait (Johnsen et al. 2009). The stability of the integron coupled with the high cost associated with carrying an active integron increase the expectations of compensatory mutations to occur which in time would reduce the burden that the integrase imposes on bacteria. As the integrase confers a higher cost (5%) than the rest of the integron (2%) the compensatory mutations would be expected to occur within the integrase gene. The integrase is transcribed from the promoter P_{int} and genetic alteration in this region would lead to lower expression levels of the integrase. It is therefore tempting to speculate that attempts by the bacterial cells to reduce the biological cost would occur in the promoter region of the integrase gene.

2.0 Caveats and speculations

2.1 Transformation by electroporation

Transformation by electroporation did not yield any transformants. This was unexpected as the method has been found to efficiently introduce plasmid DNA into *E.coli* (Hanahan et al. 1995). Efforts to determine the source of the problem yielded no results as the *E.coli* cells used in this experiment were found to be of good quality and that the control experiment

illustrated that the electroporation experiment also worked efficiently. This then led to the hypothesis that the lack of success with this approach was caused by one of the elements in that were present on the plasmid ligated with the class 1 integron. Further electroporation attempts with the pTM4 (without the integron) and the previous cloning steps in which the generated plasmids were successfully expressed in *E.coli* when they did not contain the integron narrowed down the source of problem as laying within the integron. For some unforeseen reason the introduction of the class 1 integron in *E.coli* seemed to result in non-viable cells. A search through literature relieved that reports of cell toxicity caused by other members of the tyrosine recombinase family have been made with an example of Cre protein (briefly mentioned above). The toxic effect of Cre on cells depends on the strand cleavage activity and is therefore intrinsic to its activity as a recombinase (Silver and Livingston 2001). A study into the expression of Cre under the chicken beta-actin promoter (CAG) in *E.coli* showed that significant problems were encountered for the construction of the desired clones hence the construction of the silent- self inactivated Cre in which the Cre protein was inactivated. The inactivation of the Cre protein resolved the problems with the cloning procedure (Airenne et al. 2005). Assuming that the expectations of the researchers in the study are correct and that these findings are indeed applicable other recombinases of the integrase family flanked by targeting sites, this could explain the problems encountered in this study.

2.2 The use of PCR amplified class 1 integrons as donor DNA

As the expression of the integron in *E.coli* proved futile, a different approach which omitted the use of *E.coli* was taken. In this approach the integron was amplified with phosporylated primers which could then be easily ligated to the up and down flanks. When ligated to the flanking regions and re-amplified with the flanking region primers, a construct of the integron flanked by two regions of approximately 700 bp to 750 bp (up and down flanks) that were homologous to *lifO* and *lipB* segments was obtained. As ADP1 is naturally transformable, the presence of regions of homology in the integron construct and ADP1 was expected to yield *A. baylyi* cells that contained the integron. This was however not the case as no transformants were obtained with this method. This was another unexpected outcome firstly due to the fact that the gel image (shown in figure 12) illustrated that the ligation procedure was successful thereby yielding a construct that contained sufficient regions for homologous recombination (minimum of 25 to 200bp) (Thomas and Nielsen 2005). Secondly *A. baylyi* and ADP1 in

particular is known to be highly transformable and to take up foreign DNA without any bias towards the source (Palmen et al. 1993) (Nielsen et al. 2000). It was therefore expected that this experiment would yield *A. baylyi* strain that contained the class 1 integron. The lack of success with this approach may be related to the situation that occurs when the integrase containing plasmid is introduced in *E.coli*. If indeed the integrase has toxic effects on cells, the use of PCR products in a transformation assay would imply that the transformable bacteria is exposed to a high number of integrase copies which could explain the lack of success in the transformation assays.

3.0 Loose ends

3.1 Integrase activity studies

The interesting findings related to the activity of the integrase have resulted in further studies which are aimed at determining the toxic and non toxic effect of the integrase. As a strain containing an inactive class 1 integrase has already been constructed, plans are underway to perform transformation assays with this strain with *E.coli* as an expression vector. The results from these studies would shed more light on the speculations that the class 1 integrase has similar toxic properties as the Cre protein.

3.2 Class 1 integron stability

The stability of the class 1 integrons was assessed for approximately 70 generations. This has been found to be insufficient based on the population genetic for antibiotic resistance mathematical model. Based on this model, the stability studies would have to span over at least 200 generations for any loss of resistance traits to be observed under the reported experimental conditions (screening of 1000 colonies in the end of the study). The time constraints in this study have not made it possible to conduct and complete this study. It is however expected to be conducted in the near future.

SECTION V: REFERENCES

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SECTION VI: APPENDICES

Appendix 1: Blast results from AB64.T1b sequencing

1. Sequence of the *int1*

```
>gb|GQ281659.1|  Acinetobacter baumannii strain Ab08-ColR class 1 integron integrase
(intI1) gene, complete cds
Length=1014

 Score = 1722 bits (932),  Expect = 0.0
 Identities = 939/943 (99%), Gaps = 1/943 (0%)
 Strand=Plus/Minus

Query  1    GGGCGGCAGCGCATCAAGCGGTGAGCGCACTCCGGCACCGCCAACTTTCAGCACATGCGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  996  GGGCGGCAGCGCATCAAGCGGTGAGCGCACTCCGGCACCGCCAACTTTCAGCACATGCGT

Query  61   GTAAATCATCGTCGTAGAGACGTCGGAATGGCCGAGCAGATCCTGCACGGTTCGAATGTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  936  GTAAATCATCGTCGTAGAGACGTCGGAATGGCCGAGCAGATCCTGCACGGTTCGAATGTC

Query  121  GTAACCGCTGCGGAGCAAGGCCGTCGCGAACGAGTGGCGGAGGGTGTGCGGTGTGGCGGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  876  GTAACCGCTGCGGAGCAAGGCCGTCGCGAACGAGTGGCGGAGGGTGTGCGGTGTGGCGGG

Query  181  CTTCGTGATGCCTGCTTGTTCTACGGCACGTTTGAAGGCGCGCTGAAAGGTCTGGTCATA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  816  CTTCGTGATGCCTGCTTGTTCTACGGCACGTTTGAAGGCGCGCTGAAAGGTCTGGTCATA

Query  241  CATGTGATGGCGACGCACGACACCGCTCCGTGGATCGGTCGAATGCGTGTGCTGCGCAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  756  CATGTGATGGCGACGCACGACACCGCTCCGTGGATCGGTCGAATGCGTGTGCTGCGCAAA

Query  301  AACCCAGAACCACGGCCAGGAATGCCCGGCGCGCGGATACTTCCGCTCAAGGGCGTCGGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  696  AACCCAGAACCACGGCCAGGAATGCCCGGCGCGCGGATACTTCCGCTCAAGGGCGTCGGG

Query  361  AAGCGCAACGCCGCTGCGGCCCTCGGCCTGGTCCTTCAGCCACCATGCCCGTGCACGCGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  636  AAGCGCAACGCCGCTGCGGCCCTCGGCCTGGTCCTTCAGCCACCATGCCCGTGCACGCGA

Query  421  CAGCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGCCCGATCCTTGGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  576  CAGCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGCCCGATCCTTGGA

Query  481  GCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATCCTTGACCCGCAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  516  GCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATCCTTGACCCGCAG

Query  541  TTGCAAACCCTCACTGATCCGCATGCCCGTTCCATACAGAAGCTGGGCGAACAAACGATG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  456  TTGCAAACCCTCACTGATCCGCATGCCCGTTCCATACAGAAGCTGGGCGAACAAACGATG

Query  601  CTCGCCTTCCAGAAAACCGAGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  CTCGCCTTCCAGAAAACCGAGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAA

Query  661  GCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  GCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCAC

Query  721  CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  CTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTT

Query  781  GCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  GCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGG

Query  841  GTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAGTTGACATTAGCCTGTTCGGT
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  156  GTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAGTTGACATAAGCCTGTTCGGT

Query  901  TCGTAAACTGTAATGCAAGTAGCGTATGCNNTCACGCA-CTGG  942
            |||||||||||||||||||||||||||||  ||||||| ||||
Sbjct  96   TCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGG  54
```

2. Sequence of the *aadB* gene

```
>emb|AJ289190.1|  Acinetobacter baumannii class 1 integron (intI1 gene for class
1 integrase and aadB gene for aminoglycoside (2') adenylyltransferase
Length=3858

 Score = 1463 bits (792),  Expect = 0.0
 Identities = 872/907 (96%), Gaps = 24/907 (2%)
 Strand=Plus/Plus

Query  1     GACTNCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCCTTGAAACGGATGAAGGCACG  60
             |||| |||||||||||||||||||||||||||||||| | ||||||||||||||||||
Sbjct  968   GACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACG  1027

Query  61    AACCCAGTTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTC  120
             |||||||| ||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1028  AACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTC  1087

Query  121   ACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1088  ACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTT  1147

Query  181   CATGGCTTGTTATGACTGttttttGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1148  CATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGC  1207

Query  241   GCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGCA  300
             |||||||||||||||||||||||||| | |            |||    | |||||||
Sbjct  1208  GCGTTACGCCGTGGGTCGATGTTTGATG-T-T------------ATG----G-AGCAGCA  1248

Query  301   ACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCGCATGGACACAACGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1249  ACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCGCATGGACACAACGC  1308

Query  361   AGGTCACATTGATACACCAAATTCTAGCTGCGGCAGATGAGCGAAATCTGCCGCTCTGGA  420
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1309  AGGTCACATTGATACACAAAATTCTAGCTGCGGCAGATGAGCGAAATCTGCCGCTCTGGA  1368

Query  421   TCGGTGGGGGCTGGGCGATCGATGCACGGCTAGGGCGTGTAACACGCAAGCACGATGATA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1369  TCGGTGGGGGCTGGGCGATCGATGCACGGCTAGGGCGTGTAACACGCAAGCACGATGATA  1428

Query  481   TTGATCTGACGTTTCCCGGCGAGAGGCGCGGCGAGCTCGAGGCAATGGTTGAAATGCTCG  540
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  1429  TTGATCTGACGTTTCCCGGCGAGAGGCGCGGCGAGCTCGAGGCAATAGTTGAAATGCTCG  1488

Query  541   GCGGGCGCGTCACGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCGGGGATGAGTTAC  600
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1489  GCGGGCGCGTCATGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCGGGGATGAGTTAC  1548

Query  601   TTGACTGCGAACCTGCTTGGTGGGCAGACGAAGCGNATGAAATCGCGGAGGCTCCGCAGG  660
             |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  1549  TTGACTGCGAACCTGCTTGGTGGGCAGACGAAGCGTATGAAATCGCGGAGGCTCCGCAGG  1608

Query  661   GCTCGTGCCCAGAGGCGGCTGAGGGCGTCATCGCTGGGCGGCCAGTCCGTTGTAACAGCT  720
             |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  1609  GCTCGTGCCCAGAGGCGGCTGAGGGCGTCATCGCCGGGCGGCCAGTCCGTTGTAACAGCT  1668

Query  721   GGGAGGCGATCATCTGGGATTACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  GGGAGGCGATCATCTGGGATTACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGC  1728

Query  781   CTACAAAGCACATAGAGTCCTACAGGCTCGCATGCACCTCACTCGGGGCGGAAAAGGTTG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1729  CTACAAAGCACATAGAGTCCTACAGGCTCGCATGCACCTCACTCGGGGCGGAAAAGGTTG  1788

Query  841   AGGCCTTGCGTGCCGCTTTCAGGTCGCGATATGCGGC-TAACA-TTCGTCCA-GC-GACG  896
             ||| |||||||||||||||||||||||||||||||| ||||| |||||||| || ||||
Sbjct  1789  AGGTCTTGCGTGCCGCTTTCAGGTCGCGATATGCGGCCTAACAATTCGTCCAAGCCGACG  1848

Query  897   C-GCTTC  902
             | |||||
Sbjct  1849  CCGCTTC  1855
```

3. Sequence of the *sul1* and *orf5*

gb|FJ172370.3| Acinetobacter baumannii strain 3208 multiple antibiotic resistance region AbaR5
Length=45478

8547..9386 /gene="sul1"
9514..10014/gene="orf5"

Score = 1003 bits (543), Expect = 0.0
Identities = 554/564 (98%), Gaps = 0/564 (0%)
Strand=Plus/Plus

```
Query  1     TTCGCAGTCGCGACGCCAGAGACCGAGGGTTAGATCATGCCTAGCATTCACCTTCCGGCC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9343  TTCGCAGTCGCGACGCCAGAGACCGAGGGTTAGATCATGCCTAGCATTCACCTTCCGGCC  9402

Query  61    GCCCGCTAGCGGACCCTGGTCAGGTTCCGCGAAGGTGGGCGCAGACATGCTGGGCTCGTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9403  GCCCGCTAGCGGACCCTGGTCAGGTTCCGCGAAGGTGGGCGCAGACATGCTGGGCTCGTC  9462

Query  121   AGGATCAAACTGCACTATGAGGCGGCGGTTCATACCGCGCCAGGGGAGCGAATGGACAGC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9463  AGGATCAAACTGCACTATGAGGCGGCGGTTCATACCGCGCCAGGGGAGCGAATGGACAGC  9522

Query  181   GAGGAGCCTCCGAACGTTCGGGTCGCCTGCTCGGGTGATATCGACGAGGTTGTGCGGCTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9523  GAGGAGCCTCCGAACGTTCGGGTCGCCTGCTCGGGTGATATCGACGAGGTTGTGCGGCTG  9582

Query  241   ATGCACGACGCTGCGGCGTGGATGTCCGCCAAGGGAACGCCCGCCTGGGACGTCGCGCGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9583  ATGCACGACGCTGCGGCGTGGATGTCCGCCAAGGGAACGCCCGCCTGGGACGTCGCGCGG  9642

Query  301   ATCGACCGGACATTCGCGGAGACCTTCGTCCTGAGATCCGAGCTCCTAGTCGCGAGTTGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9643  ATCGACCGGACATTCGCGGAGACCTTCGTCCTGAGATCCGAGCTCCTAGTCGCGAGTTGC  9702

Query  361   AGCGACGGCATCGTCGGCTGTTGCACCTTGTCGGCCGAGGATCCCGAGTTCTGGCCCGAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9703  AGCGACGGCATCGTCGGCTGTTGCACCTTGTCGGCCGAGGATCCCGAGTTCTGGCCCGAC  9762

Query  421   GCCCTCAAGGGGGAGGCCGCATATCTGCACAAGCTCGCGGTGCGACGGACACATGCGGGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9763  GCCCTCAAGGGGGAGGCCGCATATCTGCACAAGCTCGCGGTGCGACGGACACATGCGGGC  9822

Query  481   CGGGGTGTCAGCTCCGCGCTGATCGAGCCTnnnnnnnnnGCCGCGCGAACGCAGGGGTGC  540
             |||||||||||||||||||||||||| ||         ||||||||||||||||||||||
Sbjct  9823  CGGGGTGTCAGCTCCGCGCTGATCGAGGCTTGCCGCCATGCCGCGCGAACGCAGGGGTGC  9882

Query  541   GCCAAGCTGCGGCTCGACTGCCAC  564
             |||||||||||||||||||||||||
Sbjct  9883  GCCAAGCTGCGGCTCGACTGCCAC  9906
```

4. Sequence of the Down flank

>emb|CR543861.1| Acinetobacter sp. ADP1 complete genome
Length=3598621

Features in this part of subject sequence:
lipase
50S ribosomal protein L19

Score = 1155 bits (625), Expect = 0.0
Identities = 629/631 (99%), Gaps = 0/631 (0%)
Strand=Plus/Plus

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Query  199      ACTCTGTCGGTGCAGCGTATAGTCTATCCACTGAAGGTGCTGGTAAATTTAATGCAATCT  258
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Sbjct  3216591  ACTCTGTCGGTGCAGCGTATAGTCTATCCACTGAAGGTGCTGGTAAATTTAATGCAATCT  3216650

Query  259      TTCCTGCTGGCGTACCAACGACTGCATGCGGTCAAGGTGAATCTTCTGTCAATGGTGTAC  318
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Query  319      GTTATTATTCATGGAGCGGTGCTTCTCCATTAACCAATCCACTCGATCCTTCAGATTATG  378
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Query  379      GCCTGAGCTTGACCAGTGTATTTAGTGGCAAAAACAATGACGGGCTGGTACCTTCATGTA  438
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Query  439      GCAGTCACTTGGGTACAGTAATTCGAGATAATTACGTATGGAATCATCTGGATGAAGTCA  498
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Sbjct  3216831  GCAGTCACTTGGGTACAGTAATTCGAGATAATTACGTATGGAATCATCTGGATGAAGTCA  3216890

Query  499      ATCAAATTCTGGGTTTACGATCTATTTTTGCACAAGACCCCGTATCCATCTTTAGACAAC  558
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Query  559      ATGCCAATCGTCTCAAAGGTCAAAATCTATAATTAAGATGCACCAGATGTAAAAAATGCG  618
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Sbjct  3217011  CCCAATCAGGCGCATTTTTATGCTGATAAGCGAAGTTAAAATTAACCTCGTTTACGCGCT  3217070

Query  679      GGCAATTTTTCACGAATACGCGCAGCTTTACCAGACAACTCACGAAGGTAGTAAAGTTTA  738
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Query  739      GCACGACGCACGTCACCACGACGTTTCACTTCAATTTTAGCAACATCTGGTGAGTGAGTT  798
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Sbjct  3217131  GCACGACGCACGTCACCACGACGTTTCACTTCAATTTTAGCAACAACTGGTGAGTGAGTT  3217190

Query  799      TGGAAAACACGCTCAACACCAACTCCGCTAG  829
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Sbjct  3217191  TGGAAAACACGCTCAACACCAACACCGCTAG  3217221
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gb|GQ293501.1| Escherichia coli strain IncA/C2 plasmid pRYC103T24 insertion sequence IS5075 transposase (tnpA) gene, complete cds; transposon Tn21 transposase, resolvase (tnpR), and TnpM (tnpM) genes, complete cds; class 1 integron In4-like IntI1 (intI1), dihydrofolate reductase (dfrA1), AadA1 streptomycin adenylyltransferase (aadA1), QacEdelta1 (qacEdelta1), and Sul1 (sul1) genes, complete cds, insertion sequence IS6100 transposase gene, complete cds, and unknown gene; and transposon Tn1696 Urf2Y (urf2Y), MerE (merE), MerD (merD), MerA (merA), MerC (merC), MerP (merP), and MerT (merT) genes, complete cds, and MerR (merR) gene, partial cds

Length=15256

9512..9516/note="putative; orf5" 9522..10022
10046..10318/note="similar to orf6; similar to Pseudomonas aeruginosa
orf6 from Tn1696 in plasmid R1033 in INSD accession
U12338"

Score = 289 bits (156), Expect = 2e-74
Identities = 194/210 (92%), Gaps = 11/210 (5%)
Strand=Plus/Plus

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Sbjct  9906   GACTGCCACCCGAACCTGCGTGGCCTATACGAGCGGCTCGGATTCACCCACGTCGACACT  9965

Query  53     TTCAAT-CCGGCTGGGAT-CAA-CTTCATCGCAGAACGCCTAGAACTCGAAATCCAACGT  109
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Sbjct  9966   TTCAATCCCGGCTGGGATCCAACCTTCATCGCAGAACGCCTAGAACTCGAAATCTAACGT  10025

Query  110    CCGTTCGGGCATCGAGGTCCATGTCGGGGTGGGACGGGCCCGTGGCTTCAAGATCACTTG  169
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Sbjct  10026  CCGTTCGGGCATCGAGGTCCATGTCGGGGTGGGACGGGCCCGTGGCTTCAAGATCACTTG  10085

Query  170    CAGTCCGACCGCGATGTCTTGGTTGCGCGA  199
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Sbjct  10086  CAGTCCGACCGCGATGTCTTGGTTGCGCGA  10115
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Appendix 2: Buffers and reagents for DNA extraction

Table containing the buffers used in the column purified extraction of genomic DNA , their composition and storage requirements.

A table containing the reagents used in the isolation of column purified genomic DNA based on the QIAGEN Genomic DNA Kit (QIAGEN, Hilden, Germany). The composition of the buffers used in genomic DNA isolation were obtained from QIAGEN Genomic DNA Handbook. The table lists the name of the buffer, its composition and the storages conditions

	Buffer	Composition	Storage
	Buffer B1 (Bacterial lysis buffer)	50 mM Tris.Cl, pH 8.0; 50 mM EDTA, pH 8.0; 0.5% Tween®-20; 0.5% TritonX-100	2-8°C or room temperature
	Buffer B2 (Bacterial lysis Buffer)	3 M guanidine HCl; 20% tween-20	2-8°C or room temperature
	Buffer QBT	750 mM NaCl; 50 mM MOPS,pH 7.0; 15% isopropanol, 0.15% Triton X-100	2-8°C or room temperature
	Buffer QC (Wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol	2-8°C or room temperature
	Buffer QF (Elution buffer)	1.25 M NaCl; 50 mM Tris.Cl, pH 8.5; 15% isopropanol	2-8°C or room temperature
	TE	10 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0	Room temperature

Appendix 3: Reagents and solutions

1. Proteinase K-stock solution
   100 mg Proteinase K
   10 ml 50 mM Tris-CL pH 8.0
   10 mM CaC

2. 10X TBE buffer
   108 g Tris base
   55 g boric acid
   9.3 g EDTA
   ddH2O to 1 liter

3. 10X TAE buffer
   48.4g Tris
   20mL of 0.5M EDTA pH 8.0
   11.42mL Glacial Acetic Acid
Add enough MQ H2O to dissolve solids, pH with HCl to 7.6-7.8, then bring up to final volume of 1000mL.

4. TE BUFFER

   0.79 g Tris HCl
   0.19 g EDTA
   ddH2O to 0.5 liter

Appendix 4: Molecular weight standard: 1kb plus

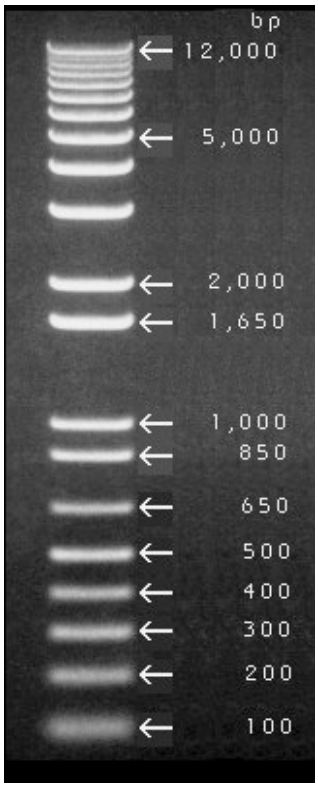

Figure 23: Gel electrophoresis of the 1 Kb Plus DNA Ladder (http://tools.invitrogen.com/content/sfs/manuals/10787018.pdf)

Appendix 5: Schematic representations of the plasmids pTM2.

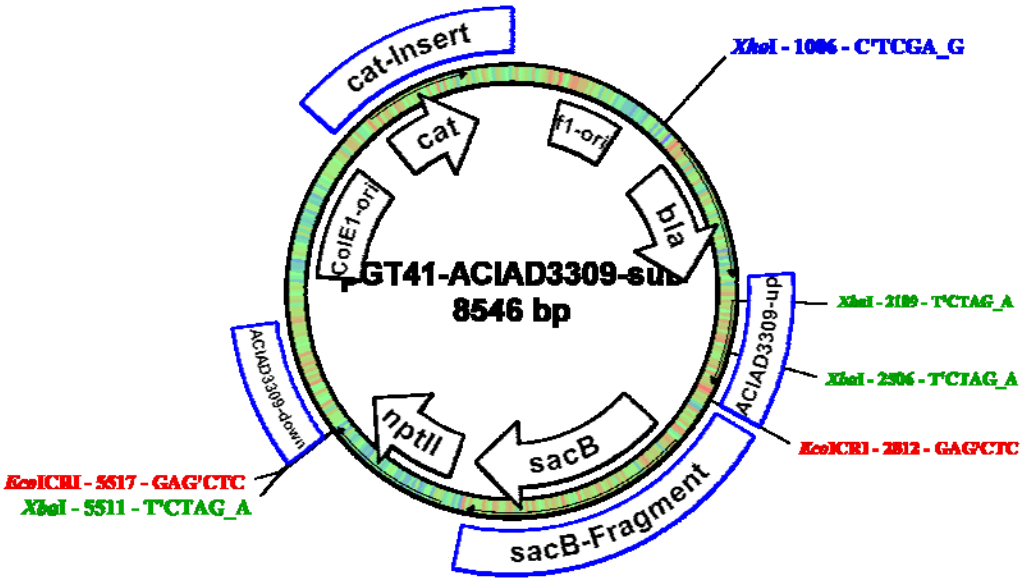

Figure 24: A schematic illustration of the structure of the plasmid pTM2 which was derived from pGT41