DIVERSITY OF CLASS 1 INTEGRONS IN AN AQUATIC ECOSYSTEM

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TABLE OF CONTENTS

SUMMARY

The emergence and rapid dissemination of antibiotic resistance is a most striking example of evolution in action, driven by lateral gene transfer. Lateral movement of genetic information is largely mediated by mobile genetic elements such as plasmids, transposons, integrons and genomic islands. These elements are able to move within or between genomes, conferring adaptive phenotypes on bacterial recipients.

Integrons are genetic elements that have a central role in the dissemination of antibiotic resistance. They are found in diverse lineages of environmental and pathogenic bacteria. Integrons possess a site specific recombination system that captures and expresses genes as a part of mobile gene cassettes. Class 1 integrons are of most clinical importance, being largely responsible for the transmission and ongoing acquisition of new antibiotic resistance genes.

The aim of this study was to investigate the evolutionary history of class 1 integrons. I also examined the potential for transmission of class 1 integrons with new gene cassettes into human pathogens and commensals and the potential for exchange of gene cassettes among different classes of integrons.

Bacteria were isolated from digestive tracts of uncooked prawns, and screened for the presence of integrons. Most prawns tested were positive for class 1 integrons. A class 1 integron recovered from a *Pseudomonas* sp. resident in the gut flora of a wild prawn had an unusual structure, and was worthy of detailed characterization. This integron was unusual, firstly in that it contained a functional Tn*402*-like transposition module, and

secondly, that it had two gene cassettes, neither of which encoded antibiotic resistance. One gene cassette was identical to a cassette previously recorded in a chromosomal class 3 integron. This structure represents an example of a key intermediate in the evolution of the clinical class 1 integron, which was previously predicted but never observed. The recovery of such an integron from a natural environment helps confirm our ideas on the likely origin of the integrons and resistance cassettes that are currently in such high abundance in clinical situations.

Another unusual class 1 integron was found in an *Acinetobacter* species isolated from the digestive flora of wild prawn. It possessed features typical of a Tn*402*-like class 1 integron, in that it contained a 3'-Conserved segment, a cassette array containing a typical *aadA2* gene cassette, and an integrase gene with 100% identity to those found in clinical contexts. These features identified it as originating from a clinical source, most likely making its way into the environment via a human waste stream. However, after its release into the natural environment, its structure had been modified by replacement of the Tn*402* terminal repeats with miniature inverted-repeat transposable elements (MITEs). It had also acquired a novel gene cassette, *msr*, that encoded methionine sulfoxide reductases. This is a function and a cassette that had not previously been recorded in any integrons.

Further work discovered multiple independent examples of this unusual class 1 integron, present in diverse *Acinetobacter* species *(Acinetobacter johnsonii*, *Acinetobacter lwoffii* and an unnamed *Acinetobacter sp.*), each of which consisted of multiple clonal lines. PCR mapping of the integron and flanking regions showed that the integron was probably part of a much larger DNA segment that was being mobilized between diverse *Acinetobacter*

strains and species.This demonstrates lateral transfer of this genetic element within and between various species of *Acinetobacter* in a marine ecosystem.

Our observations suggest the potential for rapid dissemination of class 1 integrons among various bacterial strains in aquatic environments, from where they might spread back into human pathogens and commensals. This work also shows that Tn*402*-like class 1 integrons are recruiting new gene cassettes when they are released into the environment, and that these encode phenotypes unrelated to the neutralization of antibiotics. The *msr* gene cassette, in particular, may have the potential to enhance bacterial colonization and pathogenicity because it encodes enzymes that repair oxidative damage to proteins.

The novel class 1 integrons characterized in this thesis were isolated from opportunistic pathogens (*Acinetobacter* sp. and *Pseudomonas* sp.), resident in the gut flora of prawns, which are an important food source and are consumed after only light cooking. This provides a clear pathway for these integrons to make their way back into the human commensal flora, along with any novel genes they might carry. Consequently, we should begin to think about the potential second wave of bacterial adaptation that will result from acquisition of pathogenicity and virulence factors via lateral transfer. These phenotypes will add to the considerable arsenal of resistance genes that are already present in pathogens, and will have unknown consequences for human health and welfare.

STATEMENT OF CANDIDATURE

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirement for a degree to any other university or institute other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signed

Ammara Sajjad

Date

STATEMENT OF CONTRIBUTION

This thesis contains material that has been submitted or prepared for publications, as follows:

Chapter 1: Introduction

I have done the literature review and writing of this chapter with constructive feedback from my supervisor Michael Gillings and important suggestions from my co-supervisor Hatch Stokes.

Chapter 2: Materials and methods

I have done all the writing of this chapter with constructive feedback from my supervisor Michael Gillings.

Chapter 3: Preclinical class 1 integron with a complete Tn*402*-like transposition module.

Ammara Sajjad, Marita P. Holley, Maurizio Labbate, H. W. Stokes, and Michael R. Gillings.

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My contribution to the research paper: Concept-80%, Data collection-100%, Analysis-80%, Writing 85%. Total-86.25%

Chapter 4: Mobilization of a Tn*402*-like class 1 integron with a novel cassette array via flanking miniature inverted-repeat transposable element-like structures.

Michael R. Gillings, Maurizio Labbate, Ammara Sajjad, Nellie J. Gigue`re, Marita P. Holley, and H. W. Stokes.

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My contribution to the research paper: Concept-40%, Data collection-100%, Analysis-60%, Writing 30%. Total-57.5%

I presented the poster of this paper in, International Meeting on Emerging Diseases and Surveillance (IMED) 4th -7th of February, 2011,Vienna, Austria.

Chapter 5: Evidence for a genomic island containing a class 1 integron transferring between *Acinetobacter* species in wild prawns.

Ammara Sajjad, Marita P. Holley, Piklu Roy Chowdhury, Hatch W. Stokes, and Michael R. Gillings.

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Chapter 6: Fosmid library construction and screening.

My contribution to this chapter: Concept-90%, Data collection-100%, Analysis-90%, Writing 85%. Total-91%

I received constructive help in the experimental design, data analysis and editorial assistance in the written component of this chapter from my supervisor Michael Gillings.

Chapter 7: General conclusion: The history and future of the class 1 integron.

I have done the literature review and writing of this chapter with constructive feedback from my supervisor Michael Gillings.

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CHAPTER 1 - INTRODUCTION

1.1 - Evolution and Spread of Antibiotic Resistance

Antibiotics were one of the most remarkable medical discoveries of the 20th century. Following their discovery, there was an enormously successful era in medical history during which human morbidity and mortality from bacterial infections were brought under control. Antibiotics were used for the treatment of infections in humans, animals and plants, as well as for promoting animal growth. Unfortunately, the advantage was temporary, because the intensive use and misuse of antibiotics in clinical and agricultural settings resulted in appearance of antibiotic resistance among virulent bacterial populations (Aminov, 2009; Martinez, 2009a, b). Despite ever-growing resources to combat the problem, pathogenic bacteria have continued to evolve resistance during the past decades. In addition, new opportunistic pathogens now show decreased susceptibility to antibiotics (Quinn, 1998; Weinstein *et al.*, 2005) and are becoming more and more difficult to treat, jeopardizing medical procedures which rely on antibiotic therapies to overcome infection.

It is now evident that antibiotic resistance genes date back millions or billions of years, prior to the human use of antibiotics (Hall, 2004; Martinez, 2009a, b; Allen *et al.*, 2010). Bacterial penicillinase was identified prior to the introduction of Penicillin as a therapeutic drug (Abraham and Chain, 1940). Thus it seems that as a consequence of the selection imposed by antibiotic use, some pathogens became multidrug resistant by acquiring adaptive mutations or by accumulating resistance genes from bacteria in the general environment. Contemporary studies show that the antibiotic resistance determinants found in pathogens comprise only a small portion of the total diversity of antibiotic resistance genes (Davies and Davies, 2010), and that a vast pool of antibiotic resistance genes is

present in natural environments (D'Costa *et al.*, 2006). Regardless of their functional role in non-clinical environments, it is clear that antibiotic resistance genes originate from environmental bacteria. Furthermore, antibiotic resistance determinants may provide beneficial advantages for environmental bacteria, and especially so wherever environments are impacted by pollution with antibiotic residues. In general, natural environments are important for recruitment of antibiotic resistance genes by pathogenic bacteria.

1.2 - Mechanisms of Antibiotic Resistance

The surveillance of antibiotic resistance is essential to identify the frequency and dynamics of resistance genes. The resulting data can be utilized to design strategies to manage and evaluate the impact of the growing resistance problem. Antibiotics are classified into four groups depending on their modes of activity: (i) drugs interfering with the cell wall biosynthesis; (ii) drugs interfering with nucleic acid synthesis; (iii) drugs interfering with protein biosynthesis; and iv) drugs interfering with metabolic pathways (Table 1.1) (Tenover, 2006). Each of these drug targets or pathways can be subject to a range of strategies that confer resistance. In turn, detailed knowledge of the resistance mechanisms utilized by microorganisms may inform chemical modification of antibiotic molecules to overcome resistance (Davies, 1994).

There are many mechanisms by which bacterial cells become resistant to antibiotics. These mechanisms may involve: (i) permeability changes in the cell wall of bacteria, which restrict antimicrobial access to target sites; (ii) active efflux of antibiotic molecules from the cell; (iii) target site mutation; (iv) enzymatic modification or inactivation of the antibiotic molecule; and (v) use of alternative metabolic pathways which are not inhibited by the drug (Table1.1). Each of these mechanisms is associated with a different and diverse

set of resistance genes. In some cases, resistance genes are generated by simple point mutations to existing genes within the target bacterial genome. In other cases, specific resistance genes are found in the organisms that originally synthesized the antibiotic molecule, since the host cell must be protected from the effects of its own antibiotic (Alonso *et al.*, 2001; Normark and Normark, 2002).

Table 1.1 Mechanisms of antibiotic action and resistance.

Antibiotics	**Mechanism of action of antibacterial agent**	**Mechanism of resistance to antimicrobial agent**
Beta –lactams: e.g. Pencillins, cephalosporins, monobactams, carbapenems	Interference with cell wall synthesis	Altered penicillin-binding proteins and reduce permeability
Aminoglycosides: e.g. gentamicin, tobramycin, amikacin	Protein synthesis inhibition (Bind to 30S ribosomal subunit)	Decreased ribosomal binding, reduced uptake, modifying enzymes
Fluoroquinolones: e.g. nalidixic acid, ciprofloxacin, norfloxacin	Interference with nucleic acid synthesis, Inhibit DNA synthesis	Altered DNA gyrase, reduced permeability
Folate inhibitors:e.g. trimethoprim, sulphonamides	Inhibition of metabolic pathway	Altered targets, alternative enzyme
Tetracycline	Protein synthesis inhibition (Bind to 30S ribosomal subunit)	efflux
Chloramphenicol	Protein synthesis inhibition (Bind to 50S ribosomal subunit)	acetyltransferase, exporters

Modified from (Tenover, 2006; Davies and Davies, 2010).

1.3 - Acquisition and Emergence of Resistance Genes

The acquisition and dissemination of bacterial resistance is a complex process, involving various mechanisms. Some bacterial species show high intrinsic resistance against specific antibiotics. Other species may acquire resistance phenotypes via mutation (point mutation, recombination, deletions/insertions) or via lateral transfer of resistance genes (Normark and Normark, 2002).

1.3.1 - Mutation-Driven Antibiotic Resistance

Mutation is any change in the DNA of a bacterial genome sequence (Snyder and Champness, 2003) and is a mechanism by which susceptible bacteria can acquire resistance to an antimicrobial agent. Most mutations are only effective for a specific class of antibiotic, however mutations affecting efflux or permeability commonly have a pleiotropic effect. Chromosomal gene mutations resulting in resistance to antibiotics have been described for antibiotics such as aminoglycosides e.g. streptomycin (Melancon *et al.*, 1988), aminocyclitols e.g spectinomycin (Sigmund *et al.*, 1984), fluoroquinolones and quinolones (Drlica and Zhao, 1997; Fluit *et al.*, 2001), β-lactams (Fluit *et al.*, 2001), rifampicin (Telenti *et al.*, 1993), sulfonamides, trimethoprim (Huovinen *et al.*, 1995) and tetracyclines (Ross *et al.*, 1998). Bacterial strains carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows newly resistant cells to survive and grow (Martinez and Baquero, 2000; Oliver *et al.*, 2000; Martínez-Solano *et al.*, 2008).

1.3.2 - Lateral Gene Transfer

The capability of bacteria to expand their ecological niches under strong antibiotic selective pressure can often be explained by the acquisition of resistance genes via lateral

or horizontal gene transfer. Many studies have shown that bacterial pathogens more often acquire multiple antibiotic resistance through lateral gene transfer (LGT) from an exogenous source, than through gene mutation alone (Davies, 1994; Thomas and Nielsen, 2005). Antibiotic resistance gene transfer is just one facet of microbial genome evolution driven by lateral gene transfer. Comparative analysis of complete genome sequences of Bacteria and Archaea reveal the importance of LGT in adaptation and diversification of microorganisms. The impact of lateral gene transfer is demonstrated by the fact that a significant proportion of bacterial genomes are acquired by LGT. Lateral acquisition of new DNA elements has been an important evolutionary force in microorganisms, probably over the entire history of microbial evolution (Ochman *et al.*, 2000; Beiko *et al.*, 2005; Boto, 2010).

The principal mechanisms which facilitate LGT among microorganisms are (i) transformation, (ii) transduction, and (iii) conjugation. Transformation involves the uptake and incorporation of naked DNA from the environment (Davison, 1999). Delivery of exogenous DNA to the recipient bacterial cells by phage particles or viruses is known as transduction. Some bacteriophages can integrate as a prophage into the bacterial host chromosome. Although bacteriophage-mediated transfer of resistance genes is not very common, prophages comprise a substantial part of laterally acquired DNA in many bacteria (Davison, 1999; Canchaya *et al.*, 2003). The third transfer process is conjugation, which is possibly the most frequent mechanism for transfer of resistance genes among wide variety of bacterial species and genera. In this process, mobilizable DNA molecules (plasmids, conjugative transposons) carrying resistance genes can be transferred from a donor to a recipient cell through direct cell to cell contact (Mazel and Davies, 1999).

For successful LGT, the foreign DNA must be maintained in the recipient cell by integration into the host chromosome or into other replicons such as plasmids. Integration of foreign DNA into a new host can be mediated through homologous or illegitimate recombination. For this reason, exchange of genetic information between closely related species is likely to be more successful than between distant relatives, due to the increased chance of conjugation and homologous recombination events in the former (Beiko *et al.*, 2005). Acquisition of new DNA sequences encoding potentially selectable functions provides benefits to the host cell. However, how long the acquired gene is maintained by a bacterial cell depends on its function and the strength of selection, balanced against the cost of maintaining the newly transferred DNA (Ochman *et al.*, 2000).

1.4 - Transfer through Mobile Genetic Elements

The spread of antibiotic resistance genes is frequently mediated by mobile genetic elements. These include plasmids, transposons, insertion sequence (IS) elements, genomic islands and gene cassettes integrated into integrons (Normark and Normark, 2002; Stokes and Gillings, 2011). Such mobile genetic elements allow movement of antibiotic resistance genes between locations within genomes (intracellular mobility) or between different bacterial cells (intercellular mobility).

1.4.1 - Plasmids

Transferable extrachromosomal DNA elements are often referred to as plasmids. They are common in Prokaryotes but can also occur in Eukaryotic organisms. Typical plasmids are circular, double stranded DNA molecules, able to replicate independently of the bacterial chromosome (Carattoli, 2003; Sherley *et al.*, 2004; Carattoli *et al.*, 2005). They encode functions essential for their replication, maintenance and transfer, and mechanisms that

determine their host range. Plasmids may vary in size, from those of 2 to 3 kilobases (kb) to many 100s of kb, which can be the equivalent of 10% of the host chromosome (Charlebois, 1999).

Plasmids have an uncanny ability to transfer by conjugation between distantly related microorganisms (Mazodier and Davies, 1991; Courvalin, 1994). They are significant factors in the emergence and dissemination of multiple drug resistance because they may carry genes conferring resistance to many different classes of antibiotics. Some plasmids may encode resistance for most, if not all, classes of antibiotics commonly used in clinical contexts, for example cephalosporins, fluoroquinolones and aminoglycosides (Davies and Wright, 1997; Martínez-Martínez *et al.*, 2008; Morita *et al.*, 2010). This situation can lead to the rapid emergence of multidrug resistance due to the co-mobilization of several antibiotic resistance genes. Thus major clinical problems can be created by the simultaneous acquisition of diverse resistance determinants in both pathogens and the agents that cause nosocomial infections.

1.4.2 - Insertion Sequences

Insertion sequence (IS) elements are the smallest autonomously mobile genetic elements (generally 0.7 to l.8 kb in size). They are flanked by short terminal inverted repeat sequences (l0 to 40 base pairs in size) and are widely distributed in both eukaryotic and bacterial genomes (Chandler and Mahillon, 2002) (Fig. 1.1). They are phenotypically cryptic in nature, generally only encoding genes associated with their own mobility. The insertion of IS elements can exert an impact directly, either through gene inactivation and/or influencing the expression of adjacent genes (Aubert *et al.*, 2003; Schneider and Lenski, 2004). In addition, IS can also alter the bacterial genome by a variety of genomic

rearrangements, including deletions, inversions, and duplications (Wei *et al.*, 2003; Kothapalli *et al.*, 2005; Iguchi *et al.*, 2006). Thus, it is generally assumed that IS elements make a significant contribution in bacterial genome diversification and evolution (Schneider *et al.*, 2002; Kawai *et al.*, 2006). IS elements are classified into about 20 known families (Mahillon and Chandler, 1998; Siguier *et al.*, 2006) based on several features, including the sequence of their transposases and their terminal inverted repeats. Although the mechanism of transposition varies between diverse IS families, most IS elements transpose through either nonreplicative (cut-and-paste) or replicative (copy-and paste) mechanisms (Chandler and Mahillon, 2002).

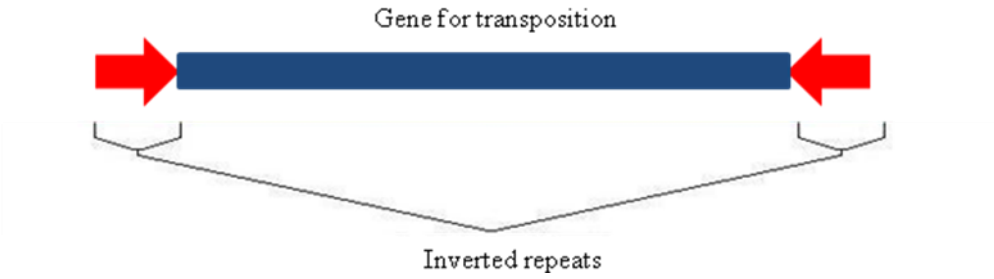

Fig. 1.1 Structure of a bacterial insertion sequence element. Insertion sequences are comprised of a region encoding proteins involved in transposition activity. Inverted repeats mark the boundaries of the insertion sequence.

1.4.3 - Transposons

Transposons, or 'jumping genes', are often responsible for the dissemination of antibiotic and heavy metal resistance genes. Transposons usually encode the transposase that catalyses transposition from one genetic location to another (McClintock, 1987; Bennett, 2004; Kidwell, 2005). Insertion of transposons may cause mutations in genes and also alter the structure of the genome. Since transposons often carry antibiotic resistance genes, they play a significant role in dissemination of antibiotic resistance (Bennett, 2008). Moreover, they are often associated with other genetic elements such as integrons and conjugative or broad-host-range plasmids, enhancing their plasticity and their dissemination among bacterial communities (Dahlberg and Hermansson, 1995; Guerra *et al.*, 2002). Transposition mechanisms generally do not have a particular preference for a target nucleotide sequence and insertion into new sites can be more or less by chance. Among different classes of transposons, class II is the one that is extensively characterized (Dahlberg and Hermansson, 1995). It is also known as the Tn*3* family and includes the Tn*21*, Tn*917*, and Tn*501* transposons (Ward and Grinsted, 1987; Dahlberg and Hermansson, 1995).

The Tn*3* family can carry resistance genes for β-lactam antibiotics including ampicillin, and other associated ESBL (Extended-Spectrum Beta Lactamase) enzymes, while Tn*21* is associated with multi-antibiotic resistance determinants such as those conferring resistance to streptomycin, spectinomycin, and sulphonamides (Zuhlsdorf and Wiedemann, 1992; Dahlberg and Hermansson, 1995; Liebert *et al.*, 1999; Bennett, 2008). Contemporary studies have described the association of transposons with some classes of integrons. For example, the Tn*21* transposon is associated with class 1 integrons and is widely distributed

in Gram-negative bacteria (Liebert *et al.*, 1999). Class 2 integrons have also been described within the Tn*7* family of transposons (Peters and Craig, 2001).

1.4.4 - Genomic Islands

Genomic islands (GIs) are mobile genetic elements located on bacterial chromosomes or plasmids. GIs are relatively large DNA segments of 10–200 kb, however segments with sizes smaller than 10 kb have also been identified and named genomic islets (Hacker and Kaper, 2000; Hentschel and Hacker, 2001). They are often inserted into tRNA genes and are flanked by 16–20-bp direct repeat structures (Hacker *et al.*, 1997) which arose as a result of their site specific integration into the target site (Dobrindt *et al.*, 2004; Schmidt and Hensel, 2004). GIs may be recognized by sequence differences between the recipient genome and the genomic island DNA, particularly in GC content, codon usage, and frequency of tetranucleotides (Karlin, 2001; Hooper and Berg, 2002). Moreover, they contain genes coding for integrases or transposases that are required for mobilizing the GI from one strain to another (Buchrieser *et al.*, 1998; Gal-Mor and Finlay, 2006). Furthermore, GI often harbour various genes which provide a selective advantage to host bacteria, thus having a significant impact on bacterial adaptation and diversification in rapidly changing environments. Based on their gene content, genomic islands have been described as resistance, pathogenicity, metabolic or fitness islands (Dobrindt *et al.*, 2004; Schmidt and Hensel, 2004).

Genomic islands may harbor a unique mobility mechanism (the type IV secretion system; T4SS) that allow them to be transferred amongst various bacterial strains (Juhas *et al.*, 2007). T4SSs are usually encoded by multiple genes organized into a single operon, necessary for the formation of a conjugative pilus and other proteins that aid transfer of the

island from donor to recipient bacteria. Moreover, they also carry bacterial effector proteins among various microorganisms, contributing towards bacterial pathogenicity. T4SSs mediate lateral gene transfer, thus contributing to genome plasticity of environmental bacterial species and evolution of bacterial pathogens by dissemination of genes encoding antibiotic resistance and virulence factors.

1.5 - Integrons

Integrons are a gene capture and expression system, originally discovered through comparison of DNA sequences flanking a broad variety of different antibiotic resistance genes on plasmids in human pathogens (Cameron *et al.*, 1986; Sundström *et al.*, 1988; Stokes and Hall, 1989; Martinez and de la Cruz, 1990; Hall and Collis, 1995; Leverstein-van Hall *et al.*, 2003). The integron system is remarkably versatile in its ability to exchange and stockpile a wide range of genes by site-specific recombination. Their ability to capture and express diverse gene cassettes provides host cells with increased fitness and the ability to rapidly adapt to the unpredictable flux of ecological niches (Rowe-Magnus and Mazel, 2001).

Integrons are comprised of various components including: (i) the *intI* gene, encoding a site-specific recombinase (Collis *et al.*, 1993); (ii) a recombination site (*attI*) for the site-specific insertion of incoming gene cassettes (Partridge *et al.*, 2000); and (iii) a promoter, Pc, to ensure expression of inserted gene cassettes (Lévesque *et al.*, 1994). Integrons are capable to generating arrays of genes of enormous diversity by repeated site-specific integration of different gene cassettes (Stokes and Hall, 1989) (Fig. 1.2).

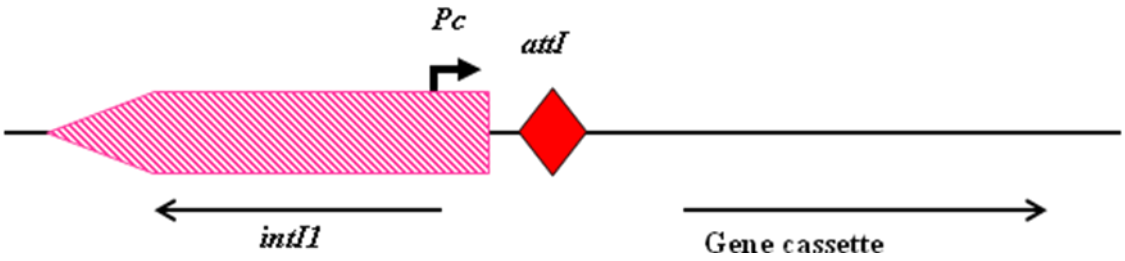

Fig. 1.2 The general structure of an integron. The *intI* gene (broad arrow) encodes the integrase, which mediates site-specific insertion of gene cassettes at the *attI* site (represented by a red diamond). On insertion, expression of gene cassettes is driven by a promoter, Pc. Arrows are indicative of the direction of transcription.

Gene cassettes are small non-replicating mobile DNA elements, consisting of an open reading frame (ORF), encoding diverse functions, and a recombination site, termed a 59-base element (59-be) (Cameron *et al.*, 1986), or *attC* site (Rowe-Magnus and Mazel, 1999). Gene cassettes usually lack a promoter and vary considerably in length due to the wide range of genes they carry (Recchia and Hall, 1995a; Partridge *et al.*, 2009). Gene cassettes may be excised from, or integrated into an integron by site-specific recombination between two sites, one in the integron (*attI*) and one in the gene cassette (*attC*) (Collis and Hall, 1992a; Collis and Hall, 1992b; Collis *et al.*, 1993) (Fig. 1.3).

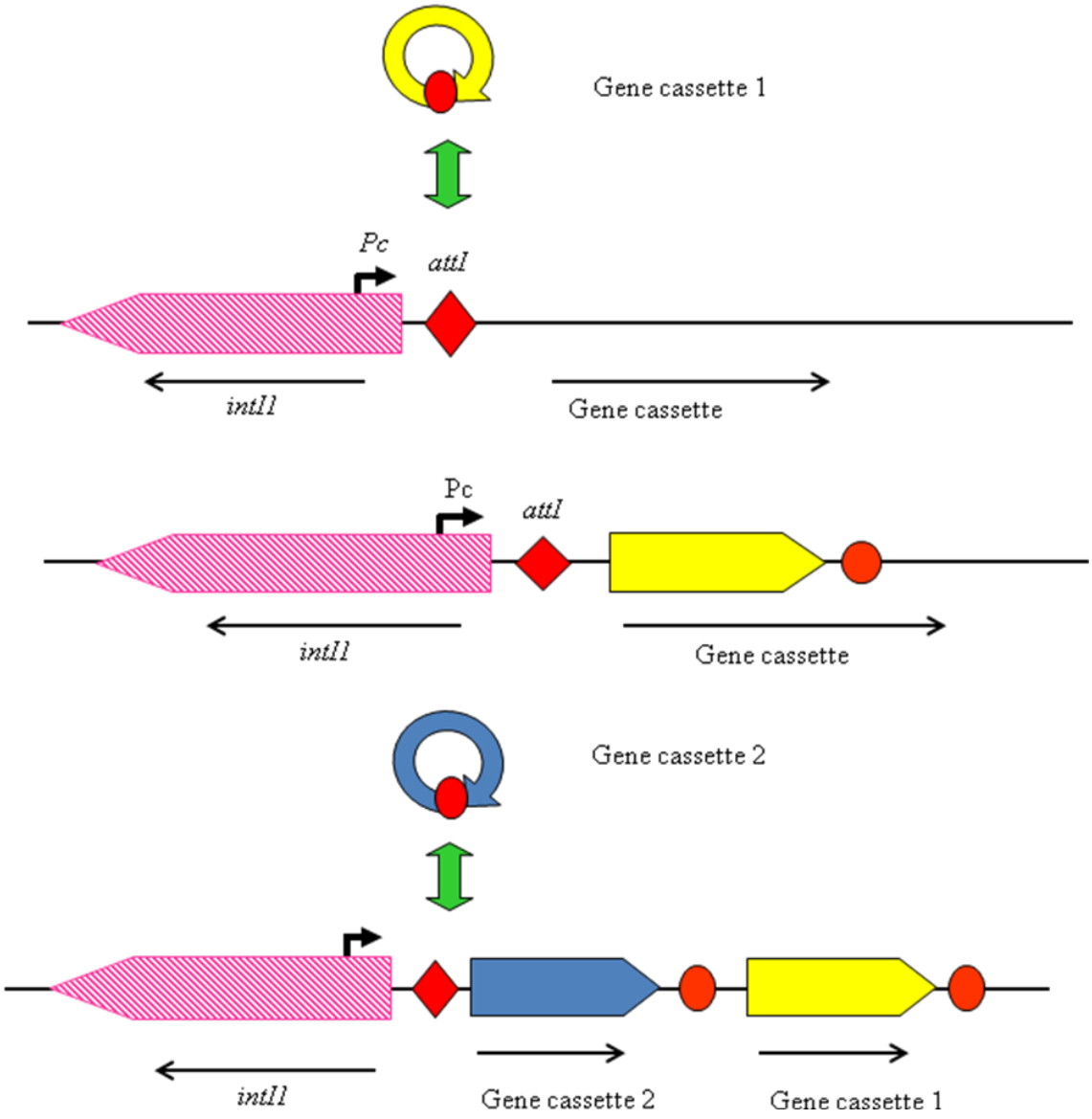

Fig. 1.3 Schematic representation of integrase mediated acquisition and excision of gene cassettes. Gene cassettes are shown in free circular forms (yellow and blue) with *attC* (red oval). The first cassette (gene cassette 1) is integrated by *attC* x *attI* recombination. The integron-integrase mediates this site-specific recombination between the primary recombination site *attI* and the secondary recombination site, *attC*, on the gene cassette. Once a cassette gets inserted, expression is carried out by a promotor Pc, located in the IntI1 coding sequence.

1.5.1 - Integron Integrases

Site-specific recombination in integrons is mediated by the integron-integrase. This enzyme belongs to the site-specific tyrosine recombinase family that includes a variety of enzymes that all use a tyrosine residue as the nucleophile in DNA strand exchange reactions. Tyrosine recombinases are generally 300-400 amino acids long and contain a structurally variable N-terminus and a larger, catalytic C-terminus. Sequence alignment shows the presence of invariant residues in conserved motifs known as box I and II. These residues are arginine (R) in box I, two histidines (H), an arginine (R) and a tyrosine (Y) in box II (Fig. 1.4). In addition to these boxes, three other patches (I, II and III) are possibly involved in the secondary structure of IntI (Nunes-Düby *et al.*, 1998) while a lysine residue in patch II is also of catalytic importance (Messier and Roy, 2001). These residues are involved in various functions. For instance, arginine residues are vital for stabilising the negative charge in the reaction transition state (Parsons *et al.*, 1990; Friesen and Sadowski, 1992), and the histidine residue potentially acts as a receptor for the proton from the attacking tyrosine residue (Grindley *et al.*, 2006). The conserved tyrosine residue is responsible for the esterification of 3' phosphoryl group and a fifth conserved residue, lysine, may act as an acid by donating a proton to the leading 5'-hydroxyl during the cleavage reaction (Krogh and Shuman, 2000).

Integron integrases have a conserved stretch of an additional 16-36 amino acids (Messier and Roy, 2001; Recchia and Sherratt, 2002) that distinguishes them from other tyrosine recombinases (Fig. 1.4). This region is located near patch III in the C-terminal catalytic domain and appears to have a role in the recognition of the *attI* and *attC* sites (Messier and Roy, 2001).

Contemporary studies have described more than 100 different types of integron-integrases based on amino acid sequence homology, and this number continues to grow as more integron-integrases are characterized (Mazel, 2006; Boucher *et al.*, 2007; Boucher *et al.*, 2009; Stokes and Gillings, 2011). The numbers originally assigned to each integron-integrase corresponded to sequence homology groups, but this classification is not used for any integrases other than those with clinical relevance, mainly due to the expanding knowledge of integron-integrase diversity. Nominally, integron-integrase genes with more than 98% nucleotide homology belong to the same integron class (Collis *et al.*, 2002a). For chromosomal integrons, integrases are often named with their host initials, for example VchIntIA from the *Vibrio cholerae* genome. All integron-integrases described to date are about 34-94 % identical or 57-96 % similar based on pairwise comparisons of IntI proteins (Collis *et al.*, 2002a).


IntI2 ----------MSNSPFLNSIRTDMRQKGYALKTEKTYLHWIKRFILF----HK-KR 41
IntI9 ----------MTRSPFLESIRQVMRTKHYSIQTEKTYLLWIKRFILF----NK-KQ 41
IntI1 MK----TATAPLPPLRSVKVLDQLRERIRYLHYSLRTEQAYVHWVRAFIRF----HG-VR 51
IntI3 MNRYNGSAKPDWVPPRSIKLLDQVRERVRYLHYSLQTEKAYVYWAKAFVLWTARSHGGFR 60
IntI6 ----------MPAPKLLDNVRNVARLKHFSLRTERSYVYWIRRFILF----HD-KR 41
IntI7 ----------MSKLLGQTRDLIRTRHLSIRTEESYLAWIKRYILF----HR-KR 39
IntI8 ---MLTESGNSASYSNGPRLLDRLRDAIRRLHYSRRTEEAYIHWTKRFIYF----HG-KR 52
IntI4 ----------MKSQFLLSVREFMQTRYYAKKTIEAYLHWITRYIHF----HN-KK 40
IntI5 ----------MKSQFLLSISEHMQTRFYAKKTIEAYLHWITRYIYF----HN-KK 40
IntI10 ----------MKSLFLTMIKDHMYSKRYAKSTIEAYLFWIAAYIRF----NN-MQ 40
HPQTMGSEEVRLFLSSLANSRHVAINTQKIALNALAFLYNRFLQQPLGDI-DYIPASKPR 100
HPKNMGEQEVTNFLTYLAVNRQVTASTQNLALCAIVFMYKHILQRELTLLPDTIKARAPK 101
HPATLGSSEVEAFLSWLANERKVSVSTHRQALAALLFFYGKVLCTDLPWLQEIGRPRPSR 111
HPREMGQAEVEGFLTMLATEKQVAPATHRQALNALLFLYRQVLGMELPWMQQIGRPPERK 120
HPRDMAEPEIRDFLSHLASHDNVAAPTQNQALSALLFLYRDVLGIRLRYMDDIERAKRPT 101
HPSELGAQHLSAFLSHPAVKRHVSASTQNQSLSAILFLYREVLGVELDWITDVARAQRPK 99
HPSELGEAAVTSFLNYLATDRNVAAATQNQALSALLFLYKETLGMELDWLDGLVRAKRPQ 112
HPSLMGDKEVEEFLTYLAVQGKVATKTQSLALNSLSFLYKEILKTPLSLEIRFQRSQLER 100
HPSLMGDKEVELFLTHLAVNGNVAAKTQSLALNSLSFLYKEILKMPLSLEIRFQRSQLER 100
HPSSMGDTQVELYLNHLVNSQNVAQGTQAQALNALSFLYKEIIKSPLSLSLDFVKSERPR 100
RLPSVISANEVQRILQVMDTRNQVIFTLLYGAGLRINECLRLRVKDFDFDNGCITVHDGK 160
RVPSVLSHNEAMSIINQLSGSYKLMFSLLYGCGLRKAELLMLRVKDIDFESRNVYVFRGK 161
RLPVVLTPDEVVRILGFLEGEHRLFAQLLYGTGMRISEGLQLRVKDLDFDHGTIIVREGK 171
RIPVVLTVQEVQTLLSHMAGTEALLAALLYGSGLRLREALGLRVKDVDFDRHAIIVRSGK 180
RVPTVLTRSEVLALLGKLTGTFYIMTSLLYGSGLRLMECLRLRVKDLDFHYNQITIRDGK 161
RLPVAFTRAEVNAVLARLRDTTWLMASLLYGSGLRLMECVRLRVKDVDFGNHQIIVRDGK 159
RMPVVLTRDEVERLLCALDGVQWLMASLLYGTGMRLMECLRLRVKDVDFGYGQILIRDGK 172
KLPVVLTRDEIRRLLEIVDPKHQLPIKLLYGSGLRLMECMRLRVQDIDFDYGAIRIWQGK 160
KLPVVLTRDEIRRLLDVVDPKYQLPAKLLYGSGLRLMECIRLRIQDIDFDYGAIRIWQGK 160
KLPVVLTQTEVSALFKHCITKHYLACALLYGSGMRLMEVLRLRIQDIDFDYNCVRIWDGK 160
GGKSRNSLLPTRLIPAIKXLIEQARLIQQDDN-LQGVG-PSLPFALDHKYPSAYRQAAWM 218
GGKDRVKMLPEKLVEPLKLHIEKVRDLHEKDL-CEGEGKTSLPSGLARKYPYAISDFKWQ 220
GSKDRALMLPESLAPSLREQLSRARAWWLKDQ-AEGRSGVALPDALERKYPRAGHSWPWF 230
GDKDRVVMLPRALVPRLRAQLIQVRAVWGQDR-ATGRGGVYLPHALERKYPRAGESWAWF 239
GNKDRVTMLPAPLKPALELHLKRVKLLHEEDL-SGGFGEVFLPHALDRKYPNASREWAWQ 220
GAKDRVTMLPAPVEESLKRHLTRVKALHEQDV-RDGFGGVYLPFALERKYKHAEREWMWQ 218
GEKDRITMLPERLVGPLKDQMDRARRIHDTDL-REGFGEVHLPYALARKYPRAGFEWNWR 231
GGKNRTVTLAKELYPHLKEQIALAKRYYDRDLHQKNYGGVWLPTALKEKYPNAPYEFRWH 220
GGKNRTVTLAKELYPHLKEQIALAKRYYDRDLHQKNYGGVWLPTALKEKYPNAPYEFRWH 220
GGKNRVVTLAGELIPQIRTQIQLVDNYLQLDLNNPLFQGVYMPHLLRKKYPNHNKQLGWQ 220
FVFPSSTLCNHPYNGKLCRHHLHDSVARKALKAAVQKAGIVSKRVTCHTFRHSFATHLLQ 278
FIFPSSVRCKHPVDGYVCRHHLHWTSLTKKLRSAVIRSG-VQKHVTAHIFRHSFATQLLK 279
WVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAVEQAG-ITKPATPHTLRHSFATALLR 289
WVFPSAKLSVDPQTGVERRHHLFEERLNRQLKKAVVQAG-IAKHVSVHTLRHSFATHLLQ 298
YVFPAGKRSIDPRSGKQRRHHVSESVLRRVVKDAVSRAG-IAKPASCHTLRHSFATHLLE 279
YVFPASRRSRDPRSGREQRHHVAETVLQRAVKAAVRQAG-IEKPGSCHTFRHSFATHLLG 277
YVFPSRNRSADPDDGVIRRHHLDESVLQRAVRTASRVAG-ISKPVHCHTFRHSFATHLLQ 290
YLFPSFQLSLDPESDVMRRHHMNETVLQKAVRRSAQEAG-IEKTVTCHTLRHSFATHLLE 279
YLFPSFQLSLDPESDVMRRHHMNETVLQRTVRRSAQEAG-IEKTVTCHTLRHSFATHLLE 279
YLFPSYKLSIDPESKQLRRHHIDEKQLQRAVKKAAFNAH-INKHVTPHTLRHSFATHLLQ 279

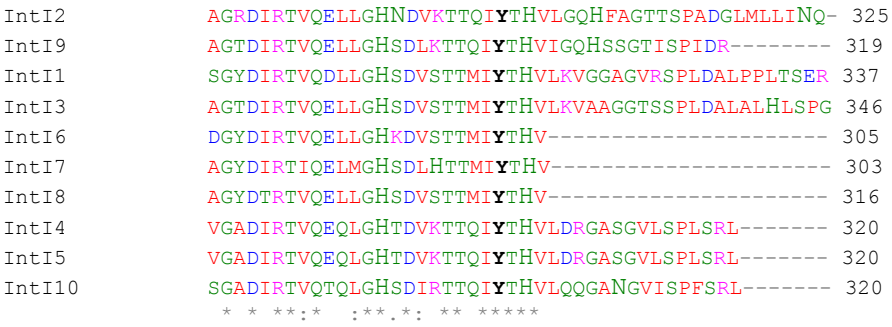

Fig. 1.4 Alignment of various IntI proteins. Residues in the boxed region represent the IntI motif (Nield *et al.*, 2001). The four very highly conserved residues from across the entire tyrosine family of recombinases (Nunes-Düby *et al.*, 1998) are shown in bold and black. GenBank accession numbers used were: IntI1, AAB59081; IntI2, AJ001816; IntI3, BAA08929; *Vibrio cholera* IntI (Vch; IntI4), AAC38424; *V. mimicus* IntI (Vmi; IntI5), AAD55407; IntI6, AAK00307; IntI7, AAK00305; IntI8, AAK00304; IntI9, AAK95987 and IntI from *V. salmonicida* plasmid pRVS1 (IntI10), CAC35342.

1.5.2 - Gene Cassettes

Gene cassettes are a distinctive mobile component of the integron system (Hall *et al.*, 1991; Recchia and Hall, 1995a). They are capable of existing as a free circular DNA molecule (Collis and Hall, 1992b), but become linear sequences when integrated into an integron, where they can be transcribed under the influence of the integron cassette promoter, Pc (Lévesque *et al.*, 1994; Collis and Hall, 1995). Usually the gene cassette contains only a single open reading frame and a 59-be (*attC)* recombination site.

Over 130 antibiotic resistance gene cassettes inserted into clinical integrons have been identified to date, where they mediate resistance to a broad spectrum of drugs, such as all known aminoglycosides, trimethoprim, β-lactams, chloramphenicol, erythromycin, streptothricin, rifampin, antiseptics and disinfectants (Recchia and Hall, 1995a; Partridge *et al.*, 2009). The most commonly identified gene cassettes are those encoding aminoglycoside adenyltransferases (e.g. *aadA1*, *aadA2*, *aadA5*, and *aadB*) and dihydrofolate reductases (e.g. *dfrA1*, *dfrA5*, *dfrA12*, *dfrA17*, and *dfrB2*). These confer resistance to aminoglycosides and trimethoprim, respectively (Levesque *et al.*, 1995; Recchia and Hall, 1995a; Partridge *et al.*, 2009). However, this preponderance of resistance cassettes is probably due to the fact that the vast majority of gene cassettes were derived from studies of antibiotic-resistant bacteria recovered from clinical contexts or ecological niches broadly exposed to antibiotics.

We know today that antibiotic resistance genes constitute only a small proportion of the gene cassettes in bacterial integrons. Several studies of non-clinical environments have recovered a wide variety of integrons carrying cassette arrays encoding diverse functions (Rowe-Magnus *et al.*, 2002; Holmes *et al.*, 2003a; Mazel, 2006). The most prevalent

functions are phage related proteins, acetyltransferases, DNA modification proteins, transport proteins, toxins, surface antigens and virulence (Clark *et al.*, 2000; Rowe-Magnus and Mazel, 2001; Stokes *et al.*, 2001; Vaisvila *et al.*, 2001). Few cassette-encoded proteins have had their function experimentally determined, but predicted functions also include restriction or methylation systems, lipases, sulphate binding proteins, polysaccharide biosynthesis and dNTP pyrophosphohydrolase (Rowe-Magnus and Mazel, 2001; Nield *et al.*, 2004; Boucher *et al.*, 2007). Cassette encoded proteins from the environmental cassette pool that had known homologues in databases also included hygromycin phosphotransferase, putative toxin-antidote systems, a PemK-like plasmid maintenance protein, an RNA methyl transferase, a thiosulfate thiotransferase, and a pyrimidine dimer DNA glycosylase (Stokes *et al.*, 2001).

Studies conducted by Koenig et al (2008) identified a wide range of novel cassette encoded proteins from four marine sediment samples taken from the vicinity of Halifax, Nova Scotia. In a further study (Koenig *et al*., 2009) unusual gene-cassettes containing genes of catabolic functions were identified from industrial waste. The majority of cassettes recovered in this study encoded novel proteins with no known homologies. The discovery of diverse gene cassettes encoding various functions from environmental contexts indicates that integrons operate as a general gene-capture system with an ancient evolutionary role in bacterial adaptation.

1.5.3 - Site-Specific Recombination

Integron linked integrases recognize two distinct types of recombination sites, the *attI* site, located upstream from the IntI gene, and the *attC* site found in the integron associated gene cassettes. In site-specific recombination, gene cassettes, which can exist in free circular

form, are inserted preferentially at the *attI* site of integrons via recombination between the *attI* and *attC*. This process is mediated by IntI integrases (Sadowski, 1986; Gellert and Nash, 1987; Collis and Hall, 1992a; Collis *et al.*, 1993; Partridge *et al.*, 2000).

attI is usually 65-70 bp long and comprised of four integrase binding sites. Two of these sites are inversely oriented and designated as R and L, which constitute the simple site and core site respectively. However, an additional two IntI binding sites are direct repeats, known as DR1 (strong binding site) and DR2 (weak binding site), and are found adjacent to the core sites (Collis *et al.*, 1998). Each of the four single binding sites contains variations of the GTTRRRY core site. A full *attI1* site is required for efficient recombination with *attC* (Recchia *et al.*, 1994; Hansson *et al.*, 1997). The direct repeat DR1 and simple site (L) are considered essential for recombination (Collis *et al.*, 1998), DR2 on the other hand, is not essential but further enhances recombination efficiency (Collis *et al.*, 2002b) (Fig. 1.5).

Fig. 1.5 Structure of a typical *attI* site. The two direct repeats (DR2 and DR1) shown by arrows, are located upstream of two inversely oriented core sites. These two core sites (L and R) constitute the simple site of *attI* and are recognised by the integrase.

The cassette-associated recombination site, *attC* is located downstream of the gene in the linear form of each gene cassette (Sundström *et al.*, 1988; Stokes and Hall, 1989; Hall *et al.*, 1991). The *attC* sites were first identified as imperfect-inverted repeat sequences. Comparison of *attC* sites reveals considerable variation in both length (from 57-141 bp) and sequence (Hall *et al.*, 1991; Recchia and Hall, 1995a). Alignments of different *attC* sites have revealed that they contain imperfect inverted repeats with two 7 bp core regions. The consensus GTTRRRY is located at the right hand end (RH) of the element and an inverse core site RYYYAAC, is sited at left hand end (LH) (Hall *et al.*, 1991; Collis and Hall, 1992a; Collis and Hall, 1992b). In addition, two more putative binding sites have been identified one is to the right of LH region and other to the left of RH core site (Stokes *et al.*, 1997). The common feature of all *attC* is the central axis of symmetry in their structure, containing two simple sites which are recognised by the integrase (Fig. 1.6).

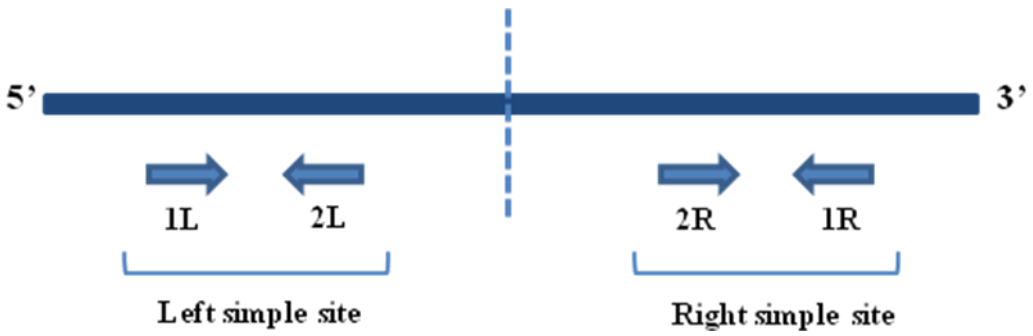

Fig. 1.6 Structure of a typical *attC* site. The left simple site consist of two core sites (1L, 2L) and likewise the right simple site (2R and 1R), shown as arrows. These core sites contain consensus sequences recognised by the integrase. The central axis of symmetry is represented by broken line.

The region between LH and RH varies both in length and sequence among *attC* sites but shows an inverted repeat structure. The difference between the LH and RH region of the *attC* potentially plays a role in ensuring correct orientation of inserted gene cassettes which then allows expression of the associated gene from Pc. The recombination point is located between the G and first T residue of the consensus sequence GTTRRRY in the 3' end of the site (Hall *et al.*, 1991). On integration of circular gene cassette, part of the *attC* ends up towards the 5' side of coding gene with which it is associated (Hall *et al.*, 1991; Collis and Hall, 1992b) thus the first six bases of each integrated cassette are derived from the cognate *attC* (Fig. 1.6).

Integron integrases (IntIs) binds strongly and specifically to the bottom strand of single-stranded *attC* site only (Francia *et al.*, 1999), folded into bulged hairpin structure (Bouvier *et al.*, 2005; Macdonald *et al.*, 2006). Formation of stem-loops structures has been proposed to be of importance for the function of the *attC* site in recombination reactions (Francia *et al.*, 1997; Francia *et al*., 1999; Hall *et al.*, 1991; Stokes *et al*., 1997; Sundström *et al*., 1988). Many studies (Johansson *et al*., 2004; Bouvier *et al*., 2005; Macdonald *et al.*, 2006) report in further detail the single-stranded recognition of *attC* and highlight the importance of the asymmetrical nucleotides within the recognition site. The folded *attC* recombines with a double stranded *attI* site or another folded *attC* site followed by replication step, which is needed to resolve the Holliday junction intermediate (Bouvier *et al*., 2005; Macdonald *et al.*, 2006).

Activity in cassette excision and integration has been demonstrated for various IntIs such as, IntI1 (Collis and Hall, 1992a; Collis *et al.*, 2001), IntI2 (Hansson *et al*., 2002), and IntI3 (Collis *et al*., 2002a). Moreover, it has been shown that IntIs can integrate and excise

diverse gene cassettes containing a variety of *attC* sites (Drouin *et al*., 2002; Leon and Roy, 2003). However, it is not understood why these enzymes can easily recognize and excise some cassettes in comparison to others, which are poorly or not excised.

1.5.4 - Recombination Events

In principle, integron integrases mediate site-specific recombination between any of two sites *attC* x *attC, attI* x *attC* and *attI* x *attI* (Collis *et al.*, 2001; Shearer and Summers, 2009). However *attI* x *attC* recombination is preferred over others (Collis and Hall, 1992a). Recent studies reported integrative recombination events with all the above combinations, whereas excisive events have only been documented when involving *attI* x *attC* and *attC* x *attC* (Hall *et al.*, 1991; Collis and Hall, 1992a; Collis and Hall, 1992b; Collis *et al.*, 1993; Collis and Hall, 2004). Excision and reintegration processes are responsible for the rearrangement of gene cassettes in an integron array (Collis and Hall, 1992a). Moreover, integron integrase genes of various classes can recognize the same *attC* sites, and as a consequence, gene cassettes can move between one class of integron and another.

1.5.5 - Expression of Gene Cassettes

On insertion of gene cassettes into an integron, expression is driven by a promoter, Pc, located within the 5'CS (Collis and Hall, 1995; Recchia and Hall, 1995b). The level of expression of an inserted gene cassette is dependent on its distance from the promoter Pc (Hanau-Berçot *et al.*, 2002), which will be higher when the inserted gene is located near Pc and vice versa (Collis and Hall, 1995). There are four versions of Pc in class 1 integrons, which exhibit differences in -35 and -10 sequences, and are designated as weak, strong, hybrid 1 and hybrid 2 promoters. The strong Pc promoter is considered as six times more effective than the *Escherichia coli tac* promoter, which is in turn more efficient than the

weak and hybrid promoters. Additionally, a second promoter P2 which complements the Pc weak promoter has also been described in various integrons. The P2 is located 119 bases downstream of Pc, and has been created by the insertion of three G residues between the pre-existing -35 and -10 regions, thus increasing the distance between these two regions from 14 to 17 nucleotides (Collis and Hall, 1995). In general, rearrangement of gene cassettes through excision and reintegration at the *attI* site can lead to higher expression of previously distal and weakly expressed genes (Rowe-Magnus and Mazel, 2001).

It is very unlikely that the expression of large arrays containing hundreds of cassettes could be mediated solely by Pc. A recent study (Michael and Labbate, 2010) showed that the majority of integron-associated gene cassettes in large arrays are expressed conditionally in response to environmental stressors by the presence of different intra-array promoters, suggesting increased adaptive capabilities of the integron/gene cassette system.

1.6 - Integron Classes

Over 100 classes of integrons have been identified on the basis of divergence in amino acid sequence. Originally integrons classes were named numerically, however, given the rapid expansion of known diversity in these genes, recent studies have adopted a scheme where names are assigned according to the species from which they are recovered, for example InPstQ for the integron from *Ps. stutzeri* Q (Holmes *et al.*, 2003b). Across known examples, integrases of various classes exhibit as little as 33% amino acid homology (Collis *et al.*, 2002b; Van Houdt *et al.*, 2012) reflecting the long evolutionary history of this family.

For several years after the discovery of integrons, only three major classes of integrons, designated class 1, class 2 and class 3, were known. These were widely distributed in clinical isolates. These integrons generally contained antibiotic resistance determinants as gene cassettes and were associated with transposons that themselves were embedded in other mobile genetic elements.

1.6.1 - Class 1 Integrons

Class 1 integrons are the most prevalent and well characterized class of integrons, first described by Stokes and Hall in 1989. They are widely disseminated in pathogens and commensals isolated from humans and livestock (Goldstein *et al.*, 2001). Class 1 integrons in clinical contexts are usually associated with a Tn*402* transposon that facilitates their mobility (Stokes *et al.*, 2006; Gillings *et al.*, 2008a). The Tn*402*-like class 1 integrons from clinical isolates have a number of characteristic features, including two conserved segments (5'-CS and 3'-CS), that flank the variable site where mobile gene cassettes are inserted. The 5'CS of class 1 integron consists of the three essential determinants as described previously, including, the *intI1* integron-integrase gene (Collis *et al.*, 1993), a promoter Pc, allowing expression of the inserted genes (Lévesque *et al.*, 1994), and an attachment site *attI1* for the site-specific insertion of gene cassettes (Partridge *et al.*, 2000). Class 1 integrons are bounded by 25-bp terminal inverted repeats, designated IRi and IRt. IRi is located at the left hand side of class 1 integron (beyond the *intI1* gene), while IRt is located downstream of the *tni* transposition module (Brown *et al.*, 1996; Partridge *et al.*, 2001).

The majority of class 1 integrons from clinical contexts now carry various modifications to the right hand end of this ancestral element, thus generating the 3' conserved segment. This

segment includes a *qacE* gene cassette truncated by incorporation of a *sulI* gene (sulphonamide resistance) and orf5. Moreover, partial deletion of transposition module (*tni*) from these elements made the transposon defective. However transposition can still occur if deleted functions are provided in *trans* (Brown *et al.*, 1996). Tn*402* transposons are well studied example of *res* hunters which are capable of targeting regions essential for replication or mobility of plasmids and transposons (Kholodii *et al.*, 1995; Petrovski and Stanisich, 2010). Consequently by targeting other mobile elements, Tn*402*-like class 1 integrons became a powerful vehicle for spreading antibiotic resistance genes. Although Tn*402*-like class 1 integrons exhibiting complete transposition (*tni*) module and lacking the 3'CS are known, they are not very common, which suggest the formation of the 3'CS occurred soon after the introduction of antibiotics in clinical contexts (Brown *et al.*, 1996) (Fig. 1.7).

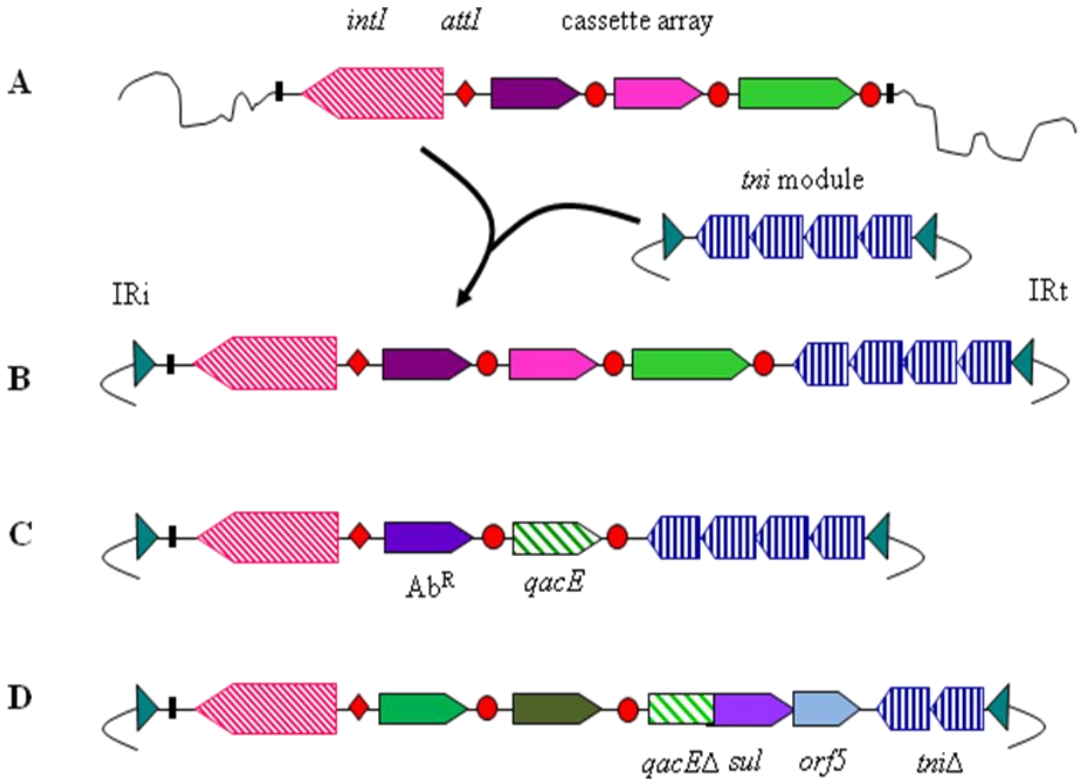

Fig. 1.7 Model for the evolutionary history of the clinical class 1 integron (A) A chromosomal class 1 integron from a *Betaproteobacterium* is captured by a Tn*402* transposon creating (B) an integron/transposon hybrid structure with enhanced ability for lateral transfer, coupled with the ability to recruit gene cassettes from the environmental cassette pool. (C) The potential ancestor of clinical class 1 integrons, containing a *qacE* gene cassette encoding resistance to quaternary ammonium compounds and complete transposition (*tni)* machinery. (D) Insertion of the *sul1* and orf5 genes, resulting in deletion of terminal part of *qacE* gene cassette. Subsequent partial deletion of the *tni* module created the 3'-CS.

1.6.2 - Class 2 Integrons

Class 2 integrons are widely distributed globally in species of Gram-negative bacteria isolated from various ecological niches. The integrase gene of the class 2 integron shows 46% sequence homology to the class 1 integrase gene (Hansson *et al.*, 2002). The key defining feature of clinical class 2 integrons is the presence of an internal stop codon in the class 2 integrase gene (Simonsen *et al.*, 1983; Hansson *et al.*, 2002). Therefore the clinical class 2 integron contains a defective integrase gene, and is unable to move gene cassettes by site-specific recombination activity.

Class 2 integrons are generally found in association with transposons, namely Tn*7* or related transposons including Tn*1825*, Tn*1826* and Tn*4132* (Hall *et al.*, 1991; Sundstrom *et al.*, 1991; Young *et al.*, 1994; Hansson *et al.*, 2002). The Tn*7*-like class 2 integron usually contains a cassette array comprised of genes *dfrA1* (confers resistance to trimethoprim), *sat2* (encoding streptothricin resistance) and *aadA1* (encoding streptomycin/spectinomycin resistance) (Sköld and Widh, 1974; Sundstrom *et al.*, 1991; White *et al.*, 2001; Yu *et al.*, 2003; Barlow *et al.*, 2004) (Fig. 1.8). Class 2 integrons harbouring other gene cassettes have also been identified including, *ereA* (resistance to erythromycin), *aadB* (resistance to gentamycin and kanamycin), *catB2* (resistance to chloramphenicol) and *estX* (putative resistance to esterase) (Biskri and Mazel, 2003; Partridge and Hall, 2005; Ramirez *et al.*, 2005).

While non-functional class 2 integrons are widely spread, studies by Marquez *et al*. have identified an active class 2 integron with an intact IntI2 gene from an *E. coli* strain in Uruguay. This differed from the Tn7-*intI2* by six nucleotides, including the internal stop codon (Marquez *et al.*, 2008). Moreover, another functional class 2 integron has been

reported in *Providencia stuartii*, isolated from cattle in Australia, where a base pair substitution encoded a glutamine instead of the stop codon found in the more usual clinical class 2 integrons (Barlow and Gobius, 2006).

Besides the functional and non-functional class 2 integrons, a hybrid class 2 integron has been recovered from *Acinetobacter baumannii* (Ploy *et al.*, 2000) which is comprised of two gene cassettes (namely, *dfrA1* and *sat* which are generally associated with class 2 integrons) and an additional 3'-CS of class 1 integrons. This significant description of a hybrid integron shows the potential for recombination between two classes of integrons.

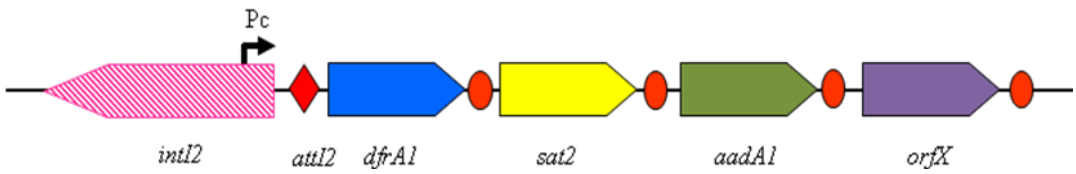

Fig. 1.8 Structure of functional class 2 integron. The *intI2* gene is shown (large striped pentagon) with an inactive integrase. The *attI2* site is represented by the red diamond. This integron contains gene cassettes, *dfrA1*, *sat2*, *aadA1* and orfX with respective *attC* which are represented by red ovals. This figure is adapted from Briski and Mazel (2003).

1.6.3 - Class 3 Integrons

Class 3 integrons were first reported in the *Serratia marcescens*, isolated from a clinical context in 1995 in Japan (Arakawa *et al.*, 1995). This class 3 integron contained two gene cassettes, *bla*IMP which encodes a metallo-beta-lactamase conferring resistance to carbapenems (Arakawa *et al.*, 1995) and *aacA4*, which confers resistance to aminoglycosides. Class 3 integrons contain a functional integrase gene encoding IntI3, which is 59% identical to IntI1 (Collis et al., 2002). Another class 3 integron was identified in *Klebsiella pneumoniae* which was isolated from an intensive care patient in Portugal (Correia *et al.*, 2003). This integron also contained two gene cassettes, containing the bla_{GES-1} gene (encoding wide spectrum β-lactamase) and the bla_{OXA-10}/*aacA4* gene (conferring resistance to kanamycin) (Correia *et al.*, 2003).

Two class 3 integrons have also been detected recently in *Delftia* species isolated from an aquatic environment in Canada (Xu *et al.*, 2007). In comparison to the clinically isolated class 3 integrons carrying antibiotic resistance genes, the environmental class 3 integrons did not contain any antibiotic resistance gene cassettes (Fig. 1.9). ORFs found in these class 3 integrons encoded proteins with unidentified functions (Xu *et al.*, 2007).

Although class 3 integrons are rarely detected and not as widespread as class 1 integrons (van Essen-Zandbergen *et al.*, 2007), they have the potential to infiltrate clinical environments and disseminate antibiotic resistance genes.

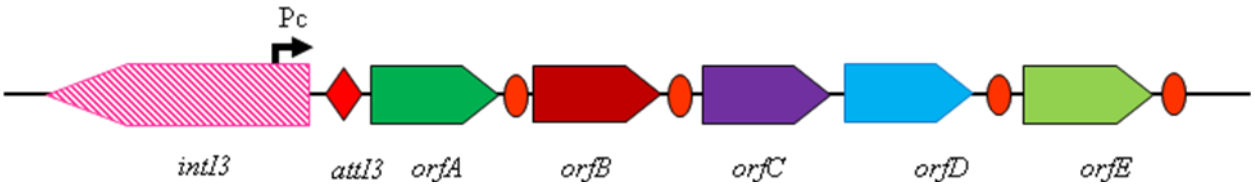

Fig. 1.9 The class 3 integron, detected in *Delftia* spp. isolated from an environmental aquatic sample. This integron carries gene cassettes *orfA*, *orfB*, *orfC*, *orfD* and *orfE* with respective *attC* which are represented by red ovals. The *IntI3* gene is shown by the striped arrow and *attI3* is illustrated by a red diamond. This figure is adapted from Xu *et al.*, 2007.

1.6.4 - Super Integrons

Chromosomal 'superintegrons' were first described in the *Vibrio cholerae* genome (Barker *et al.*, 1994; Mazel *et al.*, 1998). Since their first discovery, superintegrons have been identified in various members of the *Vibrionaceae (e.g. V. metschnikovii, V. mimicus, V. fischeri* and *V. parahaemolyticus), Pseudomonads* (e.g. *P. mendocina, P. alcaligenes* and *P. stutzeri*) and a number of genera of γ-proteobacteria (e.g. *Xanthomonas* and *Shewanella* (Rowe-Magnus *et al.*, 2001; Vaisvila *et al.*, 2001; Makino *et al.*, 2003; Rowe-Magnus *et al.*, 2003).

Initial studies showed that *Vibrio* integrons contained an integrase gene *intI4* and a large chromosomal array of gene cassettes (Mazel *et al.*, 1998) which did not encode antibiotic

resistance determinants. Subsequent studies revealed that *Vibrio* integrons can also carry genes encoding antibiotic resistance, such as the gene cassettes associated with *V. cholerae* that encode resistance to chloramphenicol acetyl transferase, glutathione transferase and fosfomycin (Heidelberg *et al.*, 2000). Although the entire repertoire of functions encoded by the cassette arrays of super-integrons are yet to be compiled, the gene cassettes are likely to encode diverse functions including DNA modification, sulphate binding activity, plasmid associated traits, transportation of smaller molecules and metabolism (Barker *et al.*, 1994; Rowe-Magnus *et al.*, 2001). The large array of gene cassettes discovered in *V. cholerae* was 126 kb long and contained at least 179 gene cassettes (Mazel *et al.*, 1998; Mazel and Davies, 1999; Rowe-Magnus *et al.*, 1999; Heidelberg *et al.*, 2000).

It has been proposed that super integrons are the ancestral structures that gave rise to multidrug resistant integrons (Rowe-Magnus *et al.*, 2001; Rowe-Magnus *et al.*, 2002; Mazel, 2006) which arose by the entrapment of the *intI* gene and linked *attI* site on mobile genetic elements e.g. transposons (Rowe-Magnus and Mazel, 1999). The large gene cassette pool associated with super integrons provides a source of novel genes that are recruited by multidrug resistant integrons (Rowe-Magnus *et al.*, 2002).

1.7 - Integron Prevalence in a Clinical Environment

Of all mobile genetic elements among Gram-negative bacteria, class 1 integrons are recognised as a major contributor to dissemination of antibiotic resistance genes in clinical settings. Integrons are predominantly found in Gram-negative bacteria from patients with hospital acquired infections such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Escherichia coli* and *Acinetobacter baumannii*, which are responsible for nosocomial outbreaks (Martinez-Freijo *et al.*, 1998; Lauretti *et al.*, 1999; Martinez-

Freijo *et al.*, 1999; Poirel *et al.*, 1999). However, antibiotic resistance integrons have also been reported in Gram-positive bacteria e.g. *Corynebacterium* species, *Mycobacteria*, and *Enterococcus faecalis* (Nešvera *et al.*, 1998; Nandi *et al.*, 2004; Shi *et al.*, 2006).

Contemporary studies have revealed the presence of integrons in human commensals and pathogens from wide geographical range worldwide. Integrons have been increasingly identified from intensive care units (ICUs) where antibiotic selective pressure may potentiate the emergence of MDR (multi drug resistant) bacteria conferring resistance to more than one antibiotic (Severino and Magalhães, 2002; Nijssen *et al.*, 2004; Iredell *et al.*, 2006; Zong *et al.*, 2008). A study undertaken in Italy showed incidence of two sequential outbreaks of multidrug-resistant *Acinetobacter baumannii* in the ICU (Zarrilli *et al.*, 2007). Class 1 integrons were persistently found in epidemic outbreak clones of *A. baumannii* during both outbreaks in the clinical environment. Pagani et al (2005) described similar results for the ICU outbreak in Italy caused by MDR *P. aeruginosa*. A recent study by Koratzanis et al (2011) reported the same class 1 integron in Gram-negative organisms in the Intensive Care Unit (ICU) in Athens, Greece. They discovered distinct clonal lines of *Klebsiella pneumoniae* and *Enterobacter cloacae* which shared the same *bla* (*VIM-1*)-containing class 1 integron structure. Similarly, two other studies showed the identification of a similar integron from two genetically distinct strains of *Enterobacter cloaceae* and two distinct isolates of *Acinetobacter baumannii* in the ICU (Kartali *et al.*, 2002; Agodi *et al.*, 2006). The presence of identical integrons in various strains of bacteria demonstrates the lateral transfer of genetic information within and between these species.

In addition to the high prevalence of integrons, many bacterial strains contain multiple integrons. Consequently they can become resistant to wide range of antibiotics (e.g. β-

lactamase, carbapenemase, aminoglycoside, chloramphenicol, rifampicin, trimethoprim and sulphonamide) (Norrby, 2005; Partridge *et al.*, 2009).

1.8 - Integron Prevalence in non-Clinical Environments

Although integrons are often associated with clinical settings, more recently the presence of integrons has also been assessed in microbiota of healthy, nonhospitalised individuals and from general environments. In recent studies, more attention has been focused on the prevalence of integrons in various strains of bacteria outside a clinical context such as bacteria isolated from healthy humans (Bailey *et al.*, 2010; Ben Sallem *et al.*, 2011), veterinary sources (Phornphisutthimas *et al.*, 2007; Machado *et al.*, 2008; Melendez *et al.*, 2010; Soufi *et al.*, 2012), food industries (Trobos *et al.*, 2008; Ahmed *et al.*, 2009a; Ahmed *et al.*, 2009b; Ahmed *et al.*, 2009c; Ahmed *et al.*, 2009d; Lynne *et al.*, 2009), wastewater treatment plants (Ferreira da Silva *et al.*, 2007; Moura *et al.*, 2007; Schlüter *et al.*, 2007; Boucher *et al.*, 2009; Li *et al.*, 2010; Xia *et al.*, 2010; Zhang *et al.*, 2010) and agricultural systems (Heuer *et al.*, 2011; Heuer *et al.*, 2012).

To understand the dissemination of integrons and resistance genes in various environments, investigations are required to identify potential hosts where antibiotic resistant microbes may reside. A number of studies have suggested the transfer of integrons between different hosts including humans and domestic animals e.g. *E. coli* and *Salmonella* isolated from food animals carry identical integrons to those found in human commensals and pathogens (Goldstein *et al.*, 2001; Schwarz *et al.*, 2001; Antunes *et al.*, 2006), domestic and wild animals (Gionechetti *et al.*, 2008; Dolejska *et al.*, 2009; Literak *et al.*, 2010; Stokes and Gillings, 2011; Gonçalves *et al.*, 2012), and in general environments e.g. identification of *E. coli* strains from remote areas including arctic birds,

soil and Peruvian Amazonas communities (Sjölund *et al.*, 2008; Bartoloni *et al.*, 2009; Berlemont *et al.*, 2011).

Several studies of integrons in non-clinical environments showed the presence of novel gene cassettes which encode functions other than antibiotic resistance, such as metabolic functions (Ahmed and Shimamoto, 2004; Labbate *et al.*, 2008). This demonstrates that integrons are a widespread modular gene packing and transfer mechanism. In addition, the discovery of diverse integron classes with non-clinical gene cassettes from soil environments further strengthens the above hypothesis (Nield *et al.*, 2001; Nield *et al.*, 2004; Stokes and Gillings, 2011).

1.9 - Integron Prevalence in the Aquatic Environment

Aquatic systems may represent an environmental reservoir where the evolution and rise of new resistance genes may occur. In addition to clinical waste, bacteria from various sources e.g. veterinary, agriculture, industrial and urban waste, are collected and mixed with environmental bacterial strains in water bodies. This may contaminate water with antibiotic resistance genes which may be then be transferred back to human populations via direct ingestion of this contaminated water, e.g. swimming in lakes, rivers and the sea or via indirect transfer of resistant bacteria through the food chain. Recently, integrons have been described in various water bodies including, river water, surface water, drinking water (Mukherjee and Chakraborty, 2006; Chen *et al.*, 2011; Figueira *et al.*, 2011; Xu *et al.*, 2011), raw and treated wastewater, industrial waste water (Tennstedt *et al.*, 2003; Gaze *et al.*, 2005; Gaze *et al.*, 2008; Figueira *et al.*, 2011; Gaze *et al.*, 2011; Pellegrini *et al.*, 2011; Girlich *et al.*, 2012) and in seafood (Ryu *et al.*, 2011; Sajjad *et al.*, 2011; Jun *et al.*, 2012). Regardless of their diverse geographical locations, bacteria containing the same

antibiotic resistance genes have been detected from different aquatic media such as, hospital and animal husbandry waste effluents, treated and untreated waste waters, surface, ground and drinking waters (Zhang *et al.*, 2009). All these data suggest that the spread of resistance determinants in natural aquatic systems together with their mobile genetic elements provides a route for newly evolved integron cassette arrays to ultimately make their way back into humans.

Culture-based approaches, while extremely useful to study and characterise integrons and gene cassettes from isolated microorganism, do not necessarily provide comprehensive information for quantifying abundance of integrons and gene cassettes among microbial communities. Due to the disparity between cultivable and *in situ* diversity, many studies have employed culture-independent molecular methods using analysis of total community DNA to study integrons (Binh *et al*., 2009; Gillings *et al*., 2008b; Heuer *et al*., 2007).

The majority of approaches used for the screening of integrons mainly rely on PCR amplification with primers targeting conserved segments that amplify across the variable region. Moreover, molecular techniques such as quantitative real time PCR and multiplex PCR have proven effective for characterising integrons and gene cassettes from environmental samples (Binh *et al*., 2009; Heuer *et al*., 2007; Dillon *et al.*, 2005). Other approaches involve the amplification of the specific *intI* gene of various integron classes and the subsequent use of specific primers to amplify the gene cassette array followed by cloning, restriction fragment length polymorphism (RFLP) analysis, sequencing and southern blot hybridisation for the identification of specific gene cassettes (Binh *et al*., 2009; Levesque *et al.*, 1995).

1.10 - Aims of the Current Research

Integrons are genetic elements which play an important role in emergence and dissemination of antibiotic resistance among bacterial populations. Class 1 integrons are considered to be a major culprit for recruiting resistance determinants into clinical environments. However, their maintenance and possible association with other mobile elements in the environment remains poorly known. Recently, class 1 integrons carrying antibiotic resistance determinants have been detected in microbial species from general environments. Considering this evidence, it is essential to expand research activities that characterize integrons and their ongoing evolution to include non-pathogenic and environmental microorganisms. Over the last few years, concerns have been raised about the potential impact of integrons that carry antibiotic resistance cassette arrays being released into aquatic ecosystems (Baquero *et al.*, 2008; Rosewarne *et al.*, 2010; Moura *et al.*, 2011; Drudge *et al.*, 2012).

Wastes from human and animal populations contain antibiotic resistance determinants which are ultimately released into aquatic ecosystems along with antibiotics and other selective agents. This has the potential to generate selection on environmental bacteria and promote the fixation of lateral transfer events in such organisms. In general, the aquatic environment is a habitat which favours lateral transfer of genetic information via mobile genetic elements (e.g. integrons, plasmids and transposons). Consequently, transfers of plasmids, integrons, and gene cassettes are likely to occur between pathogens, commensals and indigenous microorganisms.

Therefore, the overall purpose of this thesis was to study the prevalence of integrons and their inserted gene cassettes in an aquatic environment. Particular attention was given to the following:

- What is the diversity of integrons and Ab^R in selected aquatic systems and natural environments?
- Is there a potential for retransmission of class 1 integrons carrying new genes into humans?
- Is there a potential for the exchange of gene cassettes among different classes of integrons in the natural environment?
- What is the genomic context of recovered integrons?

As a model system, wild prawns were acquired from distinct geographical locations within Australia. Prawns were targeted because they filter feed on sediment, and are thus likely to acquire bacteria released from human dominated ecosystems. They are also a popular food item, and are eaten after light cooking, thus providing a ready route for retransmission into humans. Bacteria were isolated from the digestive tract of individual prawns and assessed for the presence of integrons and integrated gene cassettes. Recovered integrons and associated cassette arrays were characterized using various molecular techniques followed by phylogenetic analysis of the host bacteria to assess the guilds of lateral transfer that might exist in aquatic environments. The genomic context of integrons was determined through fosmid library construction, conjugation and southern hybridization experiments.

CHAPTER 2 - MATERIALS AND METHODS

2.1 - Source of Samples

Uncooked green prawns were acquired from retail outlets. These wild prawns were originally harvested from the ocean adjoining different Australian States, including New South Wales, Queensland and South Australia (Table 2.1). A single prawn from each location was preserved in 70% ethanol at 4^{o}C for species identification using the key of Poore (Poore, 2004). Three different species of prawns were identified, including King prawns (*Penaeus plebejus*), Banana prawns (*Penaeus merguiensis*) and School prawns (*Metapenaeus macleayi*) (Table 2.1).

2.2 - Culturing and Initial Screening

Prawns were surface sterilised in 70% ethanol. To investigate the abundance of integrons in prawn samples, culture independent approaches were carried out using direct PCRs on total community DNA of bacterial cells but failed to detect positive cells. Further work was carried out using cultivation or enrichment of bacterial cells in PC broth. For this purpose, digestive tracts were dissected from individual prawns, macerated in 200 µl of 100 mM $NaPO_4$ buffer (pH 7.4) and used to inoculate 5 ml of plate count (PC) medium (5 g tryptone, 2.5 g yeast extract and 1 g dextrose per liter). Liquid cultures were incubated at 25 ^{o}C overnight on a rotary shaker. Boiled lysate was prepared from each overnight mixed culture by harvesting 100 µl of broth, incubating at 99 ^{o}C for 10 min, snap chilling and centrifuging to pellet cell debris. Aliquots of the overnight cultures were stored at -80 ^{o}C as 25% v/v glycerol stocks.

Table 2.1 Detail of samples used in this study

Sample ID	Species Name	Common Name	Origin	*intI1* PCR[a]	*intI1* +ve Isolates[b]	*intI1* +ve/-ve Culture ID[c]
1	*Penaeus plebejus*	King Prawn	NSW	4/4	6/94	1AA12, 1BC1, 1BC11, 1BD1, 1DG10, 1DG11
2	*Penaeus plebejus*	King Prawn	Queensland	2/4	0/48	-
3	*Penaeus merguiensis*	Banana Prawn	Queensland	3/4	2/48	3AD2, 3AD12
4	*Metapenaeus macleayi*	School Prawn	NSW	1/4	0/48	-
5	*Penaeus plebejus*	King Prawn	Sth Australia	1/4	4/46	5AE3, 5AH7, 5AH8, 5AE10
6	*Penaeus plebejus*	King Prawn	Queensland	4/4	1/48	6DD4
7	*Penaeus merguiensis*	Banana Prawn	Queensland	3/4	3/46	7DG2, 7DG10, 7DG12
8	*Penaeus plebejus*	King Prawn	Queensland	3/4	0/46	-
9	*Metapenaeus macleayi*	School Prawn	NSW	4/4	0/96	-
10	*Penaeus merguiensis*	Banana Prawn	NSW	4/4	1/48	10CB3
11	*Penaeus plebejus*	King Prawn	NSW	4/4	5/142	11BE4, 11BF2, 11BF5, 11BH7, 11BF10
12	*Penaeus plebejus*	King Prawn	Queensland	4/4	4/94	12CE1, 12CE12 (IntI1+Ve) 12CE2, 12CE11 (IntI1–Ve)
13	*Penaeus plebejus*	King Prawn	Sth Australia	1/4	4/96	13BB2, 13BC4, 13BC12, 13BG2

[a] Number of prawns testing positive for the presence of a class 1 integron by *intI1* PCR on mixed cultures isolated from digestive tract of individual prawns. [b] Number of pure bacterial isolates testing positive for *intI1* by PCR. [c] Purified *intI1* positive and negative isolates used for further characterization of gene cassette and flanking regions.

Table 2.2 PCR primers used in this study

Target	Primer	Sequence (5' to 3')	Reference
intI1	HS915	CGTGCCGTGATCGAAATCCAG	(Gillings *et al.*, 2009)
	HS916	TTCGTGCCTTCATCCGTTTCC	(Gillings *et al.*, 2009)
intI1	HS463a	CTGGATTTCGATCACGGCACG	(Stokes *et al.*, 2006)
	HS464	ACATGCGTGTAAATCATCGTCG	(Stokes *et al.*, 2006)
intI2	IntI2F	CACGGATATGCGACAAAAAGGT	(Mazel *et al.*, 2000)
	IntI2R	GTAGCAAACGAGTGACGAAATG	(Mazel *et al.*, 2000)
intI3	IntI3F	GCCTCCGGCAGCGACTTTCAG	(Mazel *et al.*, 2000)
	IntI3R	ACGGATCTGCCAAACCTGACT	(Mazel *et al.*, 2000)
Cassette array	MRG284	GTTACGCCGTGGGTCGATG	(Gillings *et al.*, 2008c)
	MRG285	CCAGAGCAGCCGTAGAGC	(Gillings *et al.*, 2008c)
Cassette array	HS458	GTTTGATGTTATGGAGCAGCAACG	(Stokes *et al.*, 2006)
	HS459	GCAAAAAGGCAGCAATTATGAGCC	(Stokes *et al.*, 2006)
Integron L.H.	4G5-1F	CCCACACAATAAACGCCG	(Gillings *et al.*, 2009)
boundary	HS463a	CTGGATTTCGATCACGGCACG	(Stokes *et al.*, 2006)
Integron R.H.	4G5-1R	TGGCGATGGCTCAATGTC	(Gillings *et al.*, 2009)
boundary	4G5-tniA-F	TGCGACAAGGTACGGTAGG	(Gillings *et al.*, 2009)
msrB-aadA2	msrB-F	TGGAAGCGGAAGGCTACG	This study
	aadA2-R	AGATGGCGCTCAATGACG	This study
16SrDNA	f27	AGAGTTTGATCMTGGCTCAG	(Weisburg *et al.*, 1991)
	r1492	TACGGYTACCTTGTTACGACTT	(Weisburg *et al.*, 1991)
16S-23S IGS	1512F	GTCGTAACAAGGTAGCCGTA	(Chang *et al.*, 2005)
PCR	6R	GGGTTYCCCCRTTCRGAAAT	(Chang *et al.*, 2005)
BOX PCR	BOXA1R	CTACGGCAAGGCGACGCTGACG	(Louws *et al.*, 1994)
ERIC PCR	ERIC1	ATGTAAGCTCCTGGGGATTCAC	(Gillings and Holley, 1997)
	ERIC2	AAGTAAGTGACTGGGGTGAGCG	(Gillings and Holley, 1997)
Landmark[a] A	MRG376	GTGCTTTCACACACGAGATC	This study
	MRG377	CGCGACAAAGTTGATGTTAC	This study
Landmark B	MRG378	GCCGTGGTATCACCTACTTG	This study
	MRG379	CCGATGTTTGGTCGTAAACC	This study
Landmark C	MRG326	TTCAGCGGTGAACAGATTCC	This study
	MRG327	GCCGTTGGATCGATGTATTG	This study
Landmark D	MRG374	ACGGCGATCTGCTCTAAC	This study
	MRG375	GGCCAGACAGCAATCATG	This study
Landmark E	MRG312	GTTATACGCTGGCCAGACTGC	This study
	MRG313	TGACTTTCCGCACGAACG	This study
Landmark F	MRG324	TCCGAGATGGACGCTCTG	This study
	MRG325	GATCGGCACACGACAACC	This study
Landmark G	MRG308	GACCACCCTTATGGTTTAGTGC	This study
	MRG309	TCACCACTACCACCCTTAATCC	This study
Landmark H	4G5-F	CCCACACAATAAACGCCG	Gillings *et al.*, 2009
	HS463a	CTGGATTTCGATCACGGCACG	Stokes *et al.*, 2006
Landmark I	msrB-F	TGGAAGCGGAAGGCTACG	This study
	aadA2-R	AGATGGCGCTCAATGACG	This study
Landmark J	4G5-1R	TGGCGATGGC TCAATGTC	Gillings *et al.*, 2009
	4G5-F2	TGCGACAAGGTACGGTAGG	Gillings *et al.*, 2009
Landmark K	MRG306	ATGATGTTAGACGGCGTTCC	This study
	MRG307	TGTGGACGGATGTTTACACG	This study
Landmark L	MRG330	ATGTGGAAGCTTGGGCTTTG	This study
	MRG331	CTTGATAAAGCGCTGGAGCTG	This study
Landmark M	MRG314	GCCAAGGCCCAAATCATCC	This study
	MRG315	GTCGGCAAGGTGGGTGTTG	This study
Landmark N	MRG328	AGCCACTGAATGAGTCGTTG	This study
	MRG329	TCAGGGTAGAGCTGAGTATAGGG	This study

Boiled lysates from mixed cultures were screened for the presence of *intI1* with PCR using primers HS915 and HS916 (Marquez *et al.*, 2008) (Table 2.2). PCRs were performed in 50 µl reactions with GoTaq DNA polymerase (Promega, Madison, WI, USA) in the buffer supplied with the enzyme, 25 pmol of each primer and a final Mg concentration of 1.5 mM. PCRs were carried out using an Omn-E thermocycler (Hybaid, Middlesex, UK) with the following cycling program: 94 ^{o}C for 3 min (one cycle); 94 ^{o}C for 30 s, 65 ^{o}C for 30 s, 72 ^{o}C for 90 s (35 cycles); 72 ^{o}C for 5 min (one cycle). Positive controls were used in every amplification, either *Thauera* sp. B4 (Accession no. EU327991) which carries a chromosomal *intI1* (Gillings *et al.*, 2008a), or an *intI1* positive isolate of *Escherichia coli* (KC2) (Stokes *et al.*, 2006). PCR products were analysed using electrophoresis on 2% w/v agarose gels cast and run in TBE buffer. Gels were stained in GelRed (BIOTIUM) and photographed using Polaroid film and transmitted UV light (Yeates *et al.*, 1998).

2.3 - Recovery of *intI1* Positive Strains

Mixed cultures that were positive in the *intI1* PCR assay were used to recover pure isolates. Liquid cultures were serially diluted in 100 mM sodium phosphate buffer (pH 7.0) and 10^{-6} to 10^{-8} dilutions plated in triplicate onto PCA plates (5 g tryptone, 2.5 g yeast extract, 1 g dextrose and 15 g agar per liter). Plates were incubated at 25^{o}C for 2 days after which individual colonies were picked into sterile microtiter trays containing 100 µl of PC broth. Trays were incubated overnight at 25 ^{o}C. DNA lysates were made from individual wells containing pure bacterial isolates by harvesting 25 µl of broth into 25 µl of sterile water, heating at 99 ^{o}C for 10 min, snap chilling and centrifuging to pellet cell debris. The lysates were then used as a template for *intI1* PCR as above. Positive isolates from this screening were plated on PCA medium and plates were incubated at 25 ^{o}C to ensure purity.

A single colony was used to inoculate liquid PC medium to generate 25% v/v glycerol stocks stored at -80 ^{o}C and used as a source of genomic DNA.

2.4 - DNA Extractions

2.4.1 - Crude DNA Extraction

For PCR screening purposes, DNA was prepared from all isolates using a simple boiling method. Single colonies from overnight cultures were harvested into 100 µl sterile water followed by heating at 99 ^{o}C for 10 min to release genomic DNA. After snap chilling, the boiled preparation was centrifuged at maximum speed for 5 min to pellet cell debris. A volume of 5 µl of this boiled lysate was used as a PCR template per 50 µl reaction mixture (Stokes *et al.*, 2006).

2.4.2 - FastPrep DNA Extraction

Bacterial cells from overnight cultures were used to extract genomic DNA from *intI1* positive isolates using a bead beating method (FastPrep^{TM} Bio101) as described by Yeates and Gillings (1998). Cells were suspended in 1 ml of CLS buffer and processed in a FastPrep^{TM} homogenizer tube for 30 sec at 5.0 m/sec. Cell debris and glass beads were pelleted by centrifugation, and the resulting supernatant was mixed with an equal volume of binding matrix (diluted 1:5 with 6 M guanidine isothiocyanate). DNA bound to the matrix was collected by centrifugation. The pellet was resuspended in 800 µl of SEWS (salt ethanol wash solution: 70 % ethanol, 0.1 M sodium acetate) by vortexing, followed by centrifugation to pellet the matrix and DNA. Purified DNA was eluted from the air dried pellet with 200 µl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.6) buffer. Recovered DNA was assessed using 1% agarose electrophoresis and visualized via GelRed staining.

2.4.3 - Genomic DNA Extraction

High molecular weight DNA was extracted from pure cultures using a CTAB/phenol/chloroform method as described by Sambrook and Russell (2001). Cells (approximately 40mg) from overnight liquid cultures were pelleted using centrifugation and resuspended in 560 µl of TE buffer. Then 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added to the suspension, which was incubated for 1 hour at 37 ^{o}C. Then 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl (Cetyl trimethylammonium bromide 10 g and NaCl 4.1 g in 100 ml) solutions were added and incubated at 65 ^{o}C for another 10 min. The lysate was extracted by adding an equal volume of phenol/choloroform/isoamyl alcohol (25:24:1 v/v/v) followed by vortexing and centrifugation for 5 min. The resulting supernatant was transferred to a new tube and an equal volume of choloroform/isoamyl alcohol (24:1 v/v) was added followed by again vortexing and centrifugation for 5 min. After transferring the aqueous phase to a fresh tube, DNA was precipitated by adding 0.6 volumes of -20 ^{o}C isopropanol, and collected by centrifugation. The DNA precipitate was washed with 70% ethanol/100 mM Na acetate, air dried and resuspended in 30 µl of H_2O. RNAase treatment was performed by adding 2 µl of 1 mg/ml of RNAase A and 370 µl H_2O followed by incubation for 1 hour at 37 ^{o}C. After re-extraction with 400 ul of phenol/choloroform/isoamyl alcohol, DNA was again precipitated with 0.6 vol. isopropanol and 1/10 vol. of 3 M sodium acetate. After washing and drying, the DNA pellet was dissolved in 30 µl of H_2O. Random shearing of genomic DNA by pipetting up and down was carried out to generate approximately 40 kb fragments for use in fosmid library construction. Gel electrophoresis was conducted using 1% agarose gel to visualize the genomic DNA.

2.4.4 - Cloning and Plasmid DNA Extraction

PCR products were purified using the Wizard SV PCR purification kit (Promega) according to the manufacturer's instructions. Ligation was carried out into a TA cloning vector using a T&A cloning kit (RBC, Real Biotech Corporation) followed by transformation into TOP10 *E.coli* competent cells (Invitrogen) using a heat shock method, as per the manufacturer's protocol. Clones with inserts were identified by blue/white selection on LB agar plates containing 50 mg/ml of ampicillin, 24 mg/ml of IPTG (isopropyl beta-D-thiogalactopyranoside) and 40 mg/ml of XGal, (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). Ten white colonies were randomly picked from each sample and amplification of inserts was carried out on boiled lysates from single colonies using the original PCR primers.

Plasmids were isolated from overnight 5 ml LB broth cultures using the Wizard Miniprep DNA purification system (Promega) according to the manufacturer's instructions. Cell pellets were resuspended in 200 µl of cell resuspension solution. Cells were lysed with 250 µl of cell lysis solution, and lysates were cleared by the addition of 250 µl of neutralisation solution. After centrifugation, the supernatant, containing plasmid DNA, was mixed with 200 µl of Quantum Prep matrix in a Spin Filter column and re-centrifuged for 30 sec at 13 000 rpm. The filtrate was discarded, and the Spin Filter column was washed twice with 500 µl of column wash solution. Plasmid DNA was eluted from the Spin Filter column by addition of 100 µl of sterile water followed by centrifugation for 1 min at 13 000 rpm. The plasmid DNA was stored at -20 °C.

2.5 - PCR Amplification of Integrons and Cassette Arrays

Initially, cultures containing class 1 integrons were identified by amplification of a conserved region of *intI1* using primers HS915 and HS916 (Table 2.2) (Gillings *et al.*, 2009). DNA from all positive isolates was confirmed as *intI1* positive by a second PCR amplification targeting a 473 bp *intI1* gene fragment using primers HS463a and HS464 (Table 2) (Stokes *et al.*, 2006). To amplify Class 2 and 3 integrons, primer sets *intI2*F/*intI2*R and *intI3*F/ *intI3*R were used respectively (Table 2.2) (Mazel *et al.*, 2000). PCR cycling conditions used were: one cycle of 94 ^{o}C for 3 min; 35 cycles of 94 ^{o}C for 30 s, 60 ^{o}C (*intI1*) or 62 ^{o}C (*intI2* and *intI3*) for 30 s, 72 ^{o}C for 1 min; and one cycle of 72 ^{o}C for 5 min (Mazel *et al.*, 2000; Hardwick *et al.*, 2008). The resulting PCR products were assessed on 2% agarose gel electrophoresis.

Integrated cassette arrays were amplified using primer sets HS458 / HS459 (Stokes *et al.*, 2006; Hardwick *et al.*, 2008) and MRG284 / MRG285 (Gillings *et al.*, 2008b). All PCR amplifications were carried out in 50 µl reactions using Promega GoTaq® colourless master mix (GoTaq® Reaction Buffer (pH 8.5), 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP and 1.5 mM MgCl2). PCR cycling conditions used for cassette arrays were: one cycle of 94 ^{o}C for 3 min; 35 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 30 s, 72 ^{o}C for 1 min; and one cycle of 72 ^{o}C for 5 min (Hardwick *et al.*, 2008). The resulting PCR products were assessed on 2% agarose gel electrophoresis.

2.6 - PCR Amplifications of 16SrDNA and 16S-23S IGS Genes

16S rDNA and 16S-23S intergenic spacer region PCR amplifications were carried out for all *intI1* positive isolates using reaction mixtures as described above. The PCR amplification of 16S rDNA was carried out at 60 ^{o}C using primers f27 and r1492

(Weisburg *et al.*, 1991). 16S-23S IGS PCR was performed using primers 1512F and 6R (Chang *et al*., 2005) with following cycling conditions: 1 cycle of 94 ^{o}C for 2 min; 35 cycles of 94 ^{o}C for 1 min, 62 ^{o}C for 1 min, 72 ^{o}C for 1 min; and 1 cycle of 72 ^{o}C for 7 min (Chang *et al*., 2005). PCR products were sequenced directly after purification using Promega Wizard PCR columns. A subset of 16S-23S IGS PCR amplicons was cloned to allow unambiguous sequencing.

2.7 - RFLP Analysis of 16SrDNA

16S rDNA restriction digestion profiles were generated from all isolates. Digestion was performed using 5 µl of PCR product, 2.5 µl of 10 x buffer B (Promega), 0.5 µl of BSA (1 mg/ml) and 0.5 µl of *Hin*f1 enzyme per reaction (Promega). The digestion mixture was then incubated overnight at 37 ^{o}C. The RFLP profile of digested samples was resolved using 2% agarose gel electrophoresis. Staining and visualisation of fractionated DNA was carried out as described previously.

2.8 - BOX and ERIC PCR

Genomic fingerprints of all samples were generated using BOX and ERIC-PCR. PCR amplification was performed using Genereleaser^{TM} (Bioventures Inc.) to minimize PCR inhibitors as described by Gillings and Holley (1997). Reaction tubes contained 1 µl of target DNA along with 9 µl of Genereleaser^{TM} and two drops of sterile mineral oil. After heating in a microwave oven on high for 7 min, the tubes were incubated at 80 ^{o}C for five min before adding the master mix. The final concentration of reagents in the PCR was same as described previously with the exception that $MgCl_2$ concentration was increased to 4 mM by adding 5 µl of 25 mM $MgCl_2$ to the reaction mixture. Since BOX PCR use a single primer, 50 pmol of the BOX primer was added separately to each reaction mixture

to attain similar primer concentration used in other PCRs. The following cycling programme was used: 1 cycle of 94 ^{o}C for 3 min; 35 cycles of 94 ^{o}C for 30 s, 52 ^{o}C for 90 s, 68 ^{o}C for 8 min; and one cycle of 68 ^{o}C for 8 min (Gillings and Holley, 1997). The resultant PCR products were analysed using 2% gel electrophoresis followed by staining and visualisation as described earlier.

2.9 - Construction and Screening of Fosmid Library

Fosmid libraries of two *intI1* positive strains were constructed in order to recover entire integrons and flanking genes by using the CopyControl^{TM} Fosmid Library Production Kit (Epicentre) as described by Stokes et al., (2006). Selection of fosmids was carried out on LB agar plates containing chloramphenicol (12.5 µg/ml). Four hundred and eighty colonies were picked into 96-well plates containing 0.4 ml of LB broth with 12.5 µg/ml of chloramphenicol and incubated at 37 ^{o}C. Overnight cultures were then used to make DNA lysates by heating at 99 ^{o}C for 10 min, snap chilling and centrifuging to pellet cell debris. Screening of the fosmid library for *intI1* was carried out by using 2 µl of boiled lysate as template in PCR using primers HS463a and HS464. Purified positive clones were induced to obtain high copy number according to the instructions provided with the Kit. After induction, fosmids were extracted using a Wizard Plus SV Minipreps kit (Promega). The yield of purified fosmid was estimated by gel electrophoresis followed by staining with GelRed, and purified fosmids used for DNA sequencing.

2.10 - Conjugation Experiments

Conjugation experiments were conducted by broth mating (Phornphisutthimas *et al.*, 2007) of *intI1* positive (donor) and *intI1* negative (recipient) strains. In these experiments, NFM2 (*A. johnsonii*, streptomycin resistant) donor was mated with three recepients including *E.*

coli DH5α (nalidixic acid resistant), 12CE2 (*A. johnsonii*, rifampicin resistant) and 12CE11 (*A. lwoffii*, rifampicin resistant). The overnight cultures of donor and recipient strains were used to obtained cell pellets which were then resuspended in 500 µl of LB media. About 100 µl of donor and recipient cells were mixed in an eppendorf tube and then these cultures were grown on LB agar plates. After growing overnight at 37 ^{0}C, cultures of donor, recipient and mixed (donor & recipient) strains were diluted serially 10 fold (from 10^{-1} to 10^{-5}). These dilutions were used to spread on LB agar plates containing 25 µg/ml nalidixic acid and the appropriate selectable marker: 25 µg/ml streptomycin (for NFM2) or 50 µg/ml rifampicin (for 12EC2 and 12CE11).

2.11 - Southern Hybridization

Southern hybridization was performed as described by Southern (Southern, 1975) as modified by Sambrook and Russell (2001). Genomic DNA was extracted by bead beating (Yeates *et al.*, 1998) and plasmid DNA was prepared as described by Kado and Liu (1981). Gel electrophoresis was carried out using 0.8% agarose gels and visualized using Gel Red staining. Gel containing DNA was submerged in 250 mM HCl for 20 min for depurination followed by denaturation (0.5 M NaOH, 1.5 M NaCl) for 2 x 15 min with gentle agitation. The gel was rinsed briefly with dH_2O and submerged in two volumes of neutralization buffer (0.5 M Tris-HCl, pH7.5, 1.5 M NaCl) prior to transfer to a membrane.

DNA from the gel was transferred to a nylon membrane (HybondN+, Amersham) using a standard protocol (Sambrook and Russell, 2001). The DNA was fixed to the membrane using a UV cross linker (Stratalinker® UV Crosslinker 1800) at 1200u joules x100 for 30 sec. IntI1 digoxigenin (DIG)-labelled probes were prepared with the PCR DIG probe synthesis kit (Roche Diagnostics) using the *intI1* PCR conditions given above. The

efficiency and yield of the PCR labeling was assessed by gel electrophoresis of amplified product. The hybridization temperature was calculated according to the DIG system user's guide (Roche). The hybridization temperature used was set at 52.5°C and overnight hybridization was performed in DIG Easy-Hyb buffer.

To detect hybridized probe, membrane was incubated with Anti-Dig-Alkaline Phosphatase (AP) antibody diluted 1:10 000 in blocking solution for 30 min with gentle shaking. Unbound antibodies were removed by washing the membrane in washing buffer. After equilibrating the membrane in detection buffer for 2 min, the membrane was placed on a glass sheet and covered with CSPD chemiluminescence substrate solution diluted in 1:100 in detection buffer. Immediately the membrane was covered with cling wrap to spread the substrate evenly and without air bubbles, followed by incubation at room temperature for 5 min. The excess liquid was then removed. This membrane was incubated for 30 min at 37 ^{0}C to enhance the luminescent reaction prior to exposure to X-ray film. The membrane was re-exposed for various time periods to optimize detection.

2.12 - Preparation of PCR Products for Sequencing

PCR products were normally sequenced directly after purification using the Wizard SV PCR purification kit (Promega) according to the manufacturer's instructions. The sequencing reactions contained 5µl of purified DNA and 4 pmole of the relevant primer. Sequencing was performed at the Macquarie Sequencing Facility using dye terminator technology. Sequences were determined on an ABI 3130xl Genetic analyser using BigDye v 3.1 chemistry (Applied Biosystems). When direct sequencing of PCR products was not possible, purified PCR products were cloned as mentioned above followed by sequencing

using vector primers M13F (GTTTTCCCAGTCACGAC) and M13R (CAGTATCGACAAAGGACACACT).

2.13 - Sequence Analysis

Sequences were interrogated using bioinformatics software available through the Biomanager facility of ANGIS (http://www.angis.org.au/). BLASTN and BLASTX searches were performed through the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine nucleotide and protein homology with accessions in the existing database. Sequences were annotated by hand after performing BLAST searches for proteins and nucleotide sequences. The 16S rDNA and 16S-23S IGS sequences were aligned using CLUSTALW (Thompson *et al.*, 1994)). Phylogenetic trees were constructed from alignments using MEGA4 (Tamura *et al.*, 2007).

CHAPTER 3 – PRECLINICAL CLASS 1 INTEGRON WITH A COMPLETE Tn*402*-LIKE TRANSPOSITION MODULE

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This paper characterizes an unusual class 1 integron found in a *Pseudomonas* sp. isolated from the digestive tract of a wild prawn. This integron was the first description of a previously hypothesized intermediate in the origin of the mobile class 1 integrons that are now widespread in clinical pathogens.

In the model accounting for the origin of these distinctive structures, a Tn*402* transposon captured a chromosomal class 1 integron from the chromosome of a betaproteobacterium. The insertion of the transposon/integron hybrid into a plasmid vector then facilitated its movement between cells and species. The integron then accumulated antimicrobial resistance gene cassettes, including the *qacE* gene cassette, subsequently truncated by insertion of the *sul1* gene. Deletion of a segment of the *tni* transposition module then completed the series of events to generate the 3' conserved segment (3'CS) characteristic of many class 1 integrons in pathogens. Prior to this paper, some of the ancestral elements had been detected, including chromosomal class 1 integrons without associated resistance cassettes, and free Tn*402*-like transposons. However, a class 1 integron embedded within a Tn*402* transposon without the 3'CS or resistance gene cassettes had not been described.

This paper described such an element for the first time, and thus filled in a gap in the series of events leading to the dissemination of class 1 integrons in clinical environments. The gene cassettes in this integron did not encode antibiotic resistance determinants, but encoded hypothetical proteins. One of these cassettes was identical to a cassette previously recovered from a chromosomal class 3 integron in *Delftia*, thus demonstrating the exchange of cassettes between different classes of integrons in natural environments.

I was responsible for the design and experimental work that generated the data presented in this paper. Analysis, data interpretation and drafting of the paper was the responsibility of all authors, led by Michael Gillings and myself, who also coordinated the overall study.

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Preclinical Class 1 Integron with a Complete Tn*402*-Like Transposition Module[▽]

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The presence of integrons was assessed in gut bacteria isolated from wild-caught prawns. A pseudomonad was recovered that contained a Tn*402*-like class 1 integron with a complete transposition module and two gene cassettes. One cassette was identical to a previously described cassette from a chromosomal class 3 integron in *Delftia isaruhaensis*.

Integrons are genetic elements that contribute to lateral gene transfer. They possess a site-specific recombination system that captures and expresses genes that are found as part of mobile gene cassettes (6, 7). Integrons are comprised of an integrase gene (*intI*) whose product catalyzes the insertion of gene cassettes into a recombination site, *attI* (6, 18) (Fig. 1). Integron-associated gene cassettes exhibit great sequence diversity (1, 12, 19), but the best-known cassettes are those that contain antibiotic resistance genes.

The class 1 integrons are of great clinical importance, being responsible for the ongoing accumulation of antibiotic resistance genes (14, 15, 17, 18). There were at least two important steps leading to the generation of the basic class 1 integron backbone that now dominates clinical isolates in the antibiotic era (3, 20). The first of these was the insertion of the integron into a Tn*402*-like transposon (Fig. 1A). The second step was the generation of the 3′-conserved segment (3′-CS), involving acquisition of *sulI* and loss of part of the *tni* module (2, 18).

We are interested in the evolutionary history of these events and have shown that class 1 integrons that predate capture by Tn*402*-like transposons are common in the chromosomes of the *Betaproteobacteria* (3). The initial capture of a chromosomal class 1 integron should result in a structure that contains a complete Tn*402*-like transposition module but contains no antimicrobial resistance cassettes, other than perhaps *qacE* (5). While some class 1 integrons with complete transposition machinery have been observed in clinical contexts, they possess antibiotic resistance genes that were probably acquired some time after the initial Tn*402* capture event (10, 16).

The aim of this study was to survey natural environments for bacteria containing examples of the early transposon capture event and to assess their potential for transmitting integrons and novel gene cassettes into human pathogens. We were also interested in the potential exchange of gene cassettes among different classes of integrons. Here we describe a novel Tn*402*-like class 1 integron in a *Pseudomonas* strain recovered from a prawn, and we show that it carries unusual gene cassettes, including one previously found in a chromosomal class 3 integron.

Uncooked, wild-caught prawns (Eastern King Prawn, *Penaeus plebejus*) were collected from retail outlets in Sydney, Australia. Bacteria were cultured from individual digestive tracts of these prawns, and the resulting mixed cultures were screened for the presence of class 1, 2, and 3 integrons (4). Cultures from most prawns tested positive for class 1 integrons, but none was positive for class 2 or 3 integrons. Two hundred single colonies isolated from one mixed culture were screened for the presence of class 1 integrons. Five *intI1*-positive isolates were detected, and cassette arrays were recovered from each isolate using PCR with the primers MRG284 and MRG285 (4). Sequencing of cassette array PCR amplicons revealed an unusual structure for one of these isolates, designated 11BF10. Molecular typing of this strain was

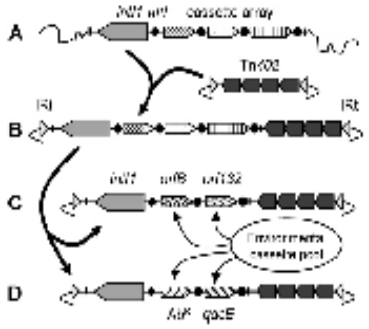

FIG. 1. Model for the origin of Tn*402* class 1 integrons. (A and B) A class 1 integron resident in the chromosome of an environmental betaproteobacterium is captured by a Tn*402* transposon (A) to generate a hybrid structure combining the ability of integrons to sample the environmental gene cassette pool with the mobility of the Tn*402* transposon (B). (C) The integron described in this article, where the hybrid structure has targeted a resolvase gene resident in a *Pseudomonas* species and the integron has acquired environmental gene cassettes, including one known to also be present in chromosomal class 3 integrons of *Delftia* (21). (D) One possible form of the ancestor of the class 1 integrons found in human pathogens and commensals. It contains an antibiotic resistance gene (Ab^R), a *qacE* gene cassette conferring resistance to quaternary ammonium compounds, and a complete *tni* transposition module. For further details, see references 20 and 3.

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FIG. 2. Schematic diagram of 16.6 kb of the class 1 integron and flanking regions in *Pseudomonas* 11BF10. Landmarks from left to right include the following: five genes for conserved hypothetical proteins; a partially deleted integrase gene (Δ*int*); a partially deleted resolvase gene (Δ*res*); a 25-bp inverted repeat (IRi) that marks the left boundary of the Tn*402*-like integron; the integron integrase gene (*intI1*) and its attendant recombination site (*attI1*); two gene cassettes, one previously found in *Delftia* (*orfB* [21]) and a novel cassette (*orf132*), separated by their respective recombination sites (*attC*); a complete *tni* transposition module composed of *tniR*, *tniQ*, *tniB*, and *tniA*; a 25-bp inverted repeat (IRt) that marks the right boundary of the integron; a toxin-antitoxin gene pair; and a set of three conserved hypothetical genes, including a TnpA repressor and two genes whose nearest homologues were found in *Delftia acidivorans*.

carried out using 16S rRNA gene sequencing, which identified it as a species of *Pseudomonas*.

To further characterize the *Pseudomonas* strain, a fosmid library was constructed (20). Five hundred fosmid clones were screened for *intI1*, resulting in three positives, which were purified and DNA sequenced by primer walking. A physical map of the class 1 integron and flanking region was then constructed (Fig. 2). The class 1 integron possessed features typical of a Tn*402*-like class 1 integron, carrying the complete *tni* transposition module and having 100% nucleotide homology to the transposition genes *tniR*, *tniQ*, *tniB*, and *tniA* from Tn*402*. The boundaries of the integron were defined by the inverted repeats IRi and IRt, identical to those in Tn*402* (8, 14, 20). We concluded that this integron represented a Tn*402*-like class 1 integron before the formation of the 3′-CS and before the loss of transposition activity (2, 18). The left-hand boundary of the Tn*402*-like integron was inserted into a partial resolvase gene, consistent with the *res*-hunting activity of this transposon (13). The other portion of the resolvase gene was not present at the right-hand boundary beyond IRt, suggesting further rearrangements after insertion, including deletions around the insertion point. One further feature of note was the presence of a toxin/antitoxin gene pair. Such genes are a common feature of chromosomal cassette arrays (1, 11), but in this case they lie outside the integron.

The class 1 integron in 11BF10 contained two gene cassettes, both with identifiable open reading frames and attendant *attC* sites. The first gene cassette exhibited 100% nucleotide identity over its entire length (756 bp) to a gene cassette (GC2, containing *orfB*) previously found in a chromosomal class 3 integron from *Delftia tsuruhatensis* A90 (21). The second cassette contained an open reading frame for which no homology could be found in existing databases. Gene cassettes for known antibiotic resistance determinants were not present, and neither was *qacE*. In previous work, we presented evidence that the ancestor of the class 1 integrons currently circulating in human pathogens arose when a chromosomal class 1 integron was captured by a Tn*402*-like transposon (3, 20). The integron described here appears to be a descendant of that original event but one that has either lost or never acquired a *qacE* cassette (5) and has been circulating in the general environment since that time. The presence of a cassette that is identical to one previously found in an environmental class 3 integron (21) demonstrates a conduit of gene transfer between class 1 and class 3 integrons in the environment. Class 1 and class 3 integron-integrases exhibit only 61% amino acid identity, but this observation suggests that both recombinases recognize the *attC* site of this cassette.

There are several points to be made from these data. The first is that this integron represents an example of a key intermediate in the evolution of the clinical class 1 integron, which was predicted but not observed until now (3). The second point is that such class 1 integrons are still circulating in the environment, where they are free to acquire gene cassettes from the enormous and diverse pool of these elements known to exist (9, 12). Third, this integron, and potentially others like it, has active transposition machinery, enabling it to autonomously target the *res* sites of diverse plasmids and potentially the Tn*21*-like transposons, thus assuming greater mobility. Finally, it was found in food that is consumed after only light cooking, giving it a ready pathway into human commensal flora, along with any novel gene cassettes it might carry.

Nucleotide sequence accession numbers. The 16S rRNA genes and fosmid sequences described in this article have been submitted to GenBank and have the accession numbers HQ423157 and HQ423158, respectively. The Tn*402*-like integron has been assigned transposon number Tn*6112* (http://www.ucl.ac.uk/eastman/tn/).

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CHAPTER 4 - MOBILIZATION OF A Tn*402*-LIKE CLASS 1 INTEGRON WITH A NOVEL CASSETTE ARRAY VIA FLANKING MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENT-LIKE STRUCTURE

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This paper characterizes an integron from *Acinetobacter johnsonii*, isolated from the digestive flora of a wild prawn. This unusual class 1 integron possessed features typical of Tn*402*-like class 1 integrons, in that it carried a typical antibiotic resistance gene cassette (*aadA2*) and a 3'-CS consisting of a *qacE*Δ*sul1* fusion and a truncated *tni* module. These features, and the identity of the *intI1* nucleotide sequence, identified it as originating from a clinical source. However, after its release into the natural environment, it had been modified by replacement of the Tn*402* terminal repeats with miniature inverted-repeat transposable elements (MITEs), and had acquired a novel gene cassette encoding methionine sulfoxide reductases. These results show that Tn*402*-like class 1 integrons can recruit new gene cassettes from the environmental gene cassette pool and acquire novel means of lateral transfer (MITEs). The presence of this integron in a popular seafood means that it may find its way back into the human commensal flora, together with a gene cassette that encodes a potential pathogenicity factor.

The publication presented in this chapter was the product of collaboration between the authors named. I was predominantly responsible for generating the primary sequence data and closing sequencing gaps. I was also involved in the data analysis and the drafting of the paper, particularly the materials and methods.

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Mobilization of a Tn*402*-Like Class 1 Integron with a Novel Cassette Array via Flanking Miniature Inverted-Repeat Transposable Element-Like Structures[▽]

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A Tn*402*-like class 1 integron was recovered from a prawn-associated bacterium. One of its cassettes included methionine sulfoxide reductase genes, the first example of such genes being captured by an integron. The integron was flanked by direct repeats that resemble miniature inverted-repeat transposable element sequences. Excision of the integron by homologous recombination through these sequences was demonstrated.

Integrons possess a site-specific recombination system that promotes dissemination of mobile gene cassettes (8, 17). About 3% of cells in the general environment contain class 1 integrons, and this integron class is broadly disseminated by lateral gene transfer (1, 6). A subset of class 1 integrons is associated with transposition functions exemplified by transposon Tn*402*, and they are commonly recovered from clinical environments. The Tn*402*-like class 1 integrons have a number of characteristic features, including two conserved segments (CS) that flank the site where mobile gene cassettes are inserted (23) (Fig. 1). The ongoing accumulation of resistance genes in Tn*402*-like integrons makes this integron subtype one of the largest contributors to the spread of multidrug resistance in human pathogens (8, 14).

Tn*402*-like class 1 integrons are also common in commensal bacteria, including those found in humans, and are spreading back into environmental bacteria (6, 12, 21, 24). Such integrons are then exposed to the highly diverse gene cassette metagenome (11), which includes a variety of potential virulence factors (1, 20). Consequently, environmental Tn*402*-like class 1 integrons may be a conduit for additional genes encoding functions other than antibiotic resistance to make their way into human pathogens. As part of a project investigating the role of animal commensal bacteria in the spread of resistance genes, we screened bacteria from the digestive tracts of prawns for the presence of class 1 integrons. Here we describe the complete DNA sequence of a novel Tn*402*-like integron and its surrounds from an *Acinetobacter* sp. strain isolated from a prawn. Digestive tracts from uncooked prawns were used to separately inoculate 5 ml plate count medium (23) and incubated for 24 h at 25°C. Four prawns, harvested from each of 16 distinct locations from Australia, the South Pacific, and Southeast Asia, were examined. More than 75% of mixed cultures from individual prawns tested positive for class 1 integrons with use of the primers HS915 and HS916 (15). PCR with these primers on pure isolates derived from such mixed cultures revealed that about 3% of 850 colonies were positive for a class 1 integron, a number consistent with findings of other studies (10, 25). We then employed a PCR method that amplified cassette arrays from Tn*402*-like class 1 integrons (13) using the primers HS458 and HS459 (10) (Fig. 1). Sequencing of one of the HS458/459 amplicons revealed an unusual structure. This unusual integron was recovered from an *Acinetobacter* sp. strain (here designated strain NFM2) isolated from a prawn (*Penaeus plebejus*) (19) harvested from ocean waters off the Australian east coast. NFM2 typing was determined by partial 16S rRNA sequencing, with the closest species match being to *Acinetobacter johnsonii*.

A fosmid library of NFM2 was constructed and an *intI1*-positive clone completely sequenced using methods previously described (23). A physical map of the relevant region is shown in Fig. 1. The integron possessed features typical of Tn*402*-derived class 1 integrons, including the presence of a 3′ CS that abutted a truncated *tni* module. However, the integron was flanked by identical copies of a 439-bp direct repeat, sequence comparisons of which are suggestive of them being miniature inverted repeat transposable elements (MITEs), a family of nonautonomous mobile elements (4). MITEs are small, nonautonomous mobile elements broadly dispersed in prokaryotes that have only recently been described (4). These elements are diverse in their sequence and properties but generally include several of the following characteristics: (i) a target site duplication, (ii) short terminal inverted repeats, (iii) high A/T content, (iv) "TA" motif at each of the termini, and (v) binding sites for host integrative factors and methyltransferases. The MITE-like elements in NFM2 include the first three of these (73% A/T). They also include at least three methyltransferase binding sites, although this domain is short (GANTC), so the significance of their presence is unclear. Many MITEs contain open reading frames, and several produce RNA transcripts, the associated secondary structure of which regulates mRNA

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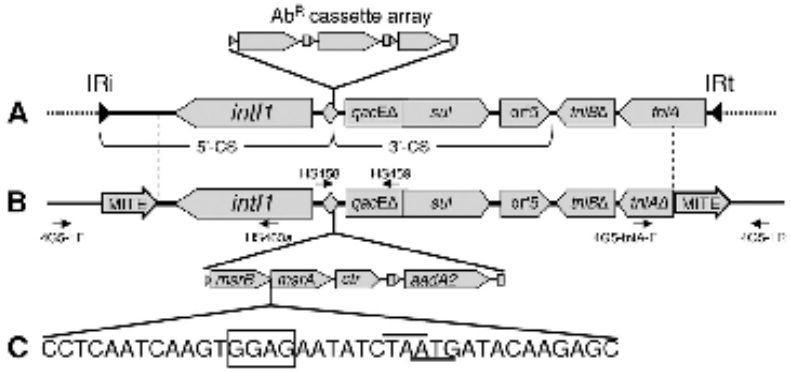

FIG. 1. Features of Tn*402*-like class 1 integrons. (A) Generalized structure of a Tn*402*-like class 1 integron as commonly recovered from multidrug-resistant pathogens. Black filled horizontal arrows indicate the inverted repeats IRi and IRt, which define the ends of Tn*402*-like transposons. The gray-shaded diamond represents the *attI1* site, at which mobile cassettes are inserted. Ab^R, antibiotic resistance. A three-cassette array is depicted here, but the type and number of cassettes are variable. The extents of the 5′ CS) and 3′ CS are indicated. (B) The vertical dashed lines define the extent of the sequence of the NFM2 integron common to that of a typical Tn*402*-like class 1 integron. The open diamond represents the *attI1* site with the structure of the two-cassette array in the NFM2 integron indicated immediately below. The small horizontal arrows indicate the relative positions of primers HS458 and HS459 and primers used to assess excision of the integron by homologous recombination between the MITE elements. (C) The sequence of the region at the end of *msrB* and the start of *msrA*. Overlined bases are the stop codon for *msrB* and the underlined bases the start codon of *msrA*. The boxed sequence is a putative ribosome binding site for *msrA*. Abbreviations: *intI1*, class 1 integron integrase gene; *qacEΔ1*, remnant of quaternary ammonium compound resistance gene; *sul*, sulfonamide resistance gene; orf5, open reading frame encoding unknown function; *tniA* and *tniB*, Tn*402*-associated transposition genes ("Δ" symbol indicates partial gene deletion). See the text for other abbreviations. The figure is not necessarily drawn to scale.

(3). The MITEs found in NFM2 do not possess obvious open reading frames. NFM2 MITE transcripts, if produced, would be likely to possess extensive secondary structures; however, we have no information as to the role, if any, in mRNA regulation.

The outer ends of the two MITE direct repeats were in turn flanked by a 5-bp direct repeat (GTTGC). We hypothesize that the MITE sequences captured the class 1 integron in a structure analogous to a composite transposon, and this has subsequently transposed to its present location. The points of insertion of the MITE-like elements are inside the normal boundary of a Tn*402*-like integron, in that the first 76 bases of the 5′ CS, including IRi, are missing at one end, and IRt and most of *tniA* are missing at the other end (Fig. 1). In silico analysis by us revealed that portions of the MITE-like sequences in NFM2 are also found in sequences derived from several *Acinetobacter* sp. clinical isolates derived from soft tissue and bloodstream infections (see reference 16 and accession numbers therein). These MITE-like examples also flank a class 1 integron at the 5′-CS segment end with the same insertion point as seen here. In addition, it has recently been found that a class 1 integron from *Enterobacter cloacae* is also flanked by MITEs, although these are not related in sequence to those found here (18). In addition, the locations of each of the MITEs at both ends of the respective integrons are different, further suggesting independent capture events.

Transposition of MITEs requires that additional functions be provided in *trans* (4). The presence of a direct repeat implies that the class 1 integron in NFM2 moved into its identified location by transposition. Indeed, transposition of the *Enterobacter cloacae* integron mobilization unit has been demonstrated (18). However, attempts to transpose the class 1 integron from NFM2 into a conjugative plasmid in a mating-out assay with an *Escherichia coli* recipient were unsuccessful (data not shown), suggesting that the required additional transposon functions are not present in this strain. However, the two copies of the MITE-like sequences are identical, and given they are oriented as direct repeats, it seemed highly likely that the integron could be readily excised from the genome by homologous recombination. To test this, PCR primers were designed to detect excision via recombination. The position of these primers is indicated in Fig. 1. Primers 4G5-1F (5′ CCC ACACAATAAACGCCG) and 4G5-1R (5′ TGGCGATGGC TCAATGTC) should generate a product of 819 bp, comprising a single MITE-like sequence remaining in the genome if the integron is deleted. Primers HS463a (11) and 4G5-tniA-F (5′ TGCGACAAGGTACGGTAGG) should recover a 1,231-bp sequence that includes the junction of the circularized, excised product. A PCR with each of these primer pairs on NFM2 genomic DNA generated PCR products of the predicted lengths (data not shown). DNA sequencing of each PCR product revealed boundaries consistent with excision via homologous recombination from a point within the MITE-like sequences.

The NFM2 integron carried two cassettes, the second of which was an *aadA2* gene cassette commonly seen in Tn*402*-like class 1 integrons. This was consistent with the fact that NFM2 was resistant to streptomycin (25 μg/ml). The first cassette was unusual for a number of reasons. It was 1,874 bases in length and included three genes. These are probably contained in a single transcript and in order are *msrB* (best match; 78% protein product identity to *Xanthobacter autotrophicus* Py2 [accession no. CP000781]), *msrA* (83% protein product identity to *Rhodopirellula baltica* SH 1 [accession no. BX294154]),

and a gene encoding a hypothetical protein (*orf255*). *msrA* and *msrB* encode methionine sulfoxide reductases, involved in the repair of proteins damaged by oxidative stress (7). The A and B forms reduce alternative isomers of methionine sulfoxide (5). The stop codon of *msrB* overlaps the start codon of *msrA* by one base (Fig. 1). The presence of a ribosome binding site immediately upstream of *msrA* suggests that this gene is translated by the process of translational coupling (9). This is the first report of *msr*-related genes being located in a mobile gene cassette. The *attC* site associated with this unusual cassette is 71 bases in length and conforms to all the criteria associated with this family of sites, including an overall imperfect repeat structure and two simple sites with the conserved domains 1R, 2R, 2L, and 1L (22). The predicted circular form of the *msr* cassette would have complementary 1R and 1L sites. We could not identify an obvious promoter in this *attC* site, so it is likely that the *aadA2* gene is expressed from the integron promoter, P_c.

Most Tn*402*-like class 1 integrons are defective transposons (2), although mobilization by transposition can still occur if the required proteins are provided in *trans*. The requirement for exogenous components limits mobilization in comparison to autonomous elements. The element found here and that seen in an *Enterobacter cloacae* isolate (18) represent two independent examples whereby class 1 integrons may be mobilized by families of elements unrelated to Tn*402*. The fact that one of these is from a clinical isolate and the other from a marine invertebrate further underscores the potential for mobilization of gene cassettes between human pathogens and bacteria found in the general environment. Finally, the NFM2 integron has an unusual cassette in that it includes three genes that may be implicated in a phenotype unrelated to the neutralization of antibiotics. This suggests that Tn*402*-like class 1 integrons are beginning to recruit new types of functions in some bacteria.

Nucleotide sequence accession numbers. The integron sequence described here is contained within the fosmid clone 4G5 and is deposited in GenBank under accession number FJ711439. Accession number GQ377756 contains the 16S rRNA sequence from NFM2.

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CHAPTER 5 – EVIDENCE FOR A GENOMIC ISLAND CONTAINING A CLASS 1 INTEGRON TRANSFERRING BETWEEN *Acinetobacter* SPECIES IN WILD PRAWNS

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This paper is an extension of the work described in Chapter 4. Our analyses from previous work showed that an unusual class 1 integron of clinical origin had made its way into *Acinetobacter* resident in prawn digestive flora. This class 1 integron contained novel gene cassettes, and was flanked by miniature inverted repeat transposable elements (MITEs).

In this paper we document the recovery of multiple independent examples of this class 1 integron from three species of *Acinetobacter (Acinetobacter johnsonii*, *Acinetobacter lwoffii* and an unnamed *Acinetobacter sp.*) each of which consisted of multiple clonal lines. This demonstrates lateral transfer of this genetic element within and between the various species of *Acinetobacter* in a marine ecosystem. Further characterization of this integron showed that it was associated with a large genomic segment common to all integron positive *Acinetobacter* strains, but absent in negative strains. The same genomic segment was identified in one clinical isolate of *Acinetobacter nosocomialis,* but without the embedded integron. These results suggest the potential for rapid dissemination of chromosomal genetic elements among various *Acinetobacter* strains in aquatic environments, from where they might spread back into human pathogens and commensals.

I was responsible for the design and experimental work that generated the data presented in this paper. Analysis, data interpretation and drafting of the paper was the responsibility of all authors, led by Michael Gillings and myself, who also coordinated the overall study.

This chapter is written as a manuscript which includes its own introduction, materials and methods and discussion. Repetition of this information was minimized as far as practically possible with respect to main introduction, materials and methods and discussion chapters. Moreover, Chapter 6 is an extension of chapter 5 which explains the methodology and techniques used to accomplish the study. Chapter 6 is a technical chapter containing detail not appropriate for the body of a journal publication. It would probably appears as on-line supplementary material

Evidence for a genomic island containing a class 1 integron transferring between *Acinetobacter* species in wild prawns

Running Title: Genomic island in *Acinetobacter* species

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Keywords: Lateral gene transfer; MITE; integron; antibiotic resistance; evolution; opportunistic pathogen

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ABSTRACT

The distribution of clinically important integrons was assessed in bacteria associated with wild prawns. A class 1 integron comprised of *aadA* and *msr* gene cassettes and flanked by MITEs (miniature inverted repeat transposable elements) was frequently identified in *Acinetobacter* species recovered from the digestive tract of wild prawns. All isolates were characterised by 16S rDNA and 16S-23S rDNA analysis, which distinguished three distinct clades, identified as *A. johnsonii, A. lwoffii* and an unnamed *Acinetobacter sp.* Fosmid libraries constructed from one isolate of *A. johnsonii* were used to examine the genomic landscape around the integron. It was concluded that it resided on a genomic island, based on PCR mapping of all isolates, and the hybridization of *intI1* probes to chromosomal, but not plasmid DNA. PCR mapping established that the same integron and extensive flanking sequences were present in the three *Acinetobacter* species. These data illustrate the potential for rapid dissemination of a novel integron via its association with an undescribed genomic island, and demonstrate the ready transmission of chromosomal genetic elements between species in aquatic ecosystems.

INTRODUCTION

Lateral gene transfer contributes to the genetic diversity of microorganisms by providing accessory genes encoding adaptive traits (Schmidt and Hensel, 2004). This lateral movement of genetic information has had a significant role in the emergence and dissemination of antibiotic resistance genes and other virulence factors among bacterial species (Chen *et al.*, 2005; Juhas *et al.*, 2008). Mobilizing genetic elements such as transposons and integrons facilitate the translocation of genes between DNA locations and between replicons (Boucher *et al.*, 2007; Gillings *et al.*, 2008). In addition, physical movement of DNA between cells is made possible by the processes of transduction, transformation and conjugation. These processes and elements acting together greatly contribute to microbial evolution and diversity (Skippington and Ragan, 2011; Gillings and Stokes, 2012).

Conjugation via autonomously replicating plasmids is recognised as a major contributor to the spread of antibiotic resistance (Smillie *et al.*, 2010). However, the genomics era has also revealed that large segments of DNA, generically known as genomic islands (GIs), can be found distributed in the chromosomes of bacterial cell lineages in a strain specific manner (Juhas *et al.*, 2009). In many cases the presence of multiple genomic islands has some cell lines possessing hundreds of kilobases of strain-specific DNA (Hayden *et al.*, 2008). Like plasmids, GIs commonly contain diverse antibiotic resistance and other virulence genes, and there is a growing realisation that GIs are important components of the mobile genome (Stokes *et al.*, 2012), particularly in *Acinetobacter* sp. (Di Nocera *et al.*, 2011).

Integrons are widely spread among diverse lineages of environmental and pathogenic bacteria (Boucher *et al.*, 2007; Cambray *et al.*, 2010). They possess a site specific recombination system which is capable of capturing and expressing diverse genes as part of mobile gene cassettes (Stokes and Hall, 1989; Collis and Hall, 1995; Hall and Collis, 1995). Class 1 integrons are major contributors to the dissemination of antibiotic resistance genes among Gram negative bacteria and they are commonly recovered from clinical environments (Partridge *et al.*, 2009). During their recent evolution, class 1 integrons became embedded in a *Tn402* transposon, and this hybrid element underwent a number of further events to generate the structure commonly found in clinical isolates. These clinical integrons commonly have a number of characteristic features including: a conserved integrase gene sequence; a conserved 3' segment (3'-CS) consisting of a truncated *qacE* gene fused with *sul1*; and a partially deleted *tni* module, rendering the transposon inactive (Partridge *et al.*, 2001; Stokes *et al.*, 2006; Gillings *et al.*, 2008).

Diverse class 1 integrons containing a 3'-CS and Tn*402*-like features are widespread in human pathogens and commensals, and are thought to have had a single ancestor, generated when an active Tn*402* transposon captured a class1 integron from the chromosome of a betaproteobacterium (Gillings *et al.*, 2008; Labbate *et al.*, 2008). These integrons are now being shed back into the environment via human waste streams where they can be detected in wild animals and in environments far removed from antibiotic use (Stokes and Gillings, 2011). In natural environments, Tn*402*-like class 1 integrons are exposed to an enormous and highly diverse pool of gene cassettes (Holmes *et al.*, 2003; Boucher *et al.*, 2007). They may recruit gene cassettes encoding adaptive functions other than antibiotic resistance, which then provide their bacterial host with a selective advantage (Holmes *et al.*, 2003; Gillings *et al.*, 2009). If such cassettes encode virulence or

pathogenicity factors, acquisition of the modified integron by existing or emerging pathogens may pose a significant human health risk.

In previous work we detected an unusual Tn*402-like* class 1 integron in a strain of *Acinetobacter johnsonii* isolated from wild prawns. The presence of a 3'-CS and truncated *tni* module argued for a clinical origin of the element, and that after its release into the environment it acquired an unusual gene cassette encoding methionine sulfoxide reductases, a potential pathogenicity factor (Grimaud *et al.*, 2001). The integron was also flanked by miniature inverted repeat transposable elements (MITEs). These MITEs were located inside the region bounded by the transposon associated inverted repeats IRi and IRt, such that translocation of the integron was now driven by the MITEs rather than the Tn*402* transposition functions (Gillings *et al.*, 2009). The presence of a novel integron potentially encoding a pathogenicity gene, and its presence in a member of a bacterial genus that is a significant opportunistic pathogen was worthy of further investigation. In this paper we have surveyed wild prawns for class 1 integrons and demonstrated the ready lateral transfer of a genomic segment containing a class 1 integron between strains and species of *Acinetobacter* that inhabit the prawn gut.

MATERIALS AND METHODS

Source of Samples

Uncooked eastern king prawns (*Penaeus plebejus*) and banana prawns (*Penaeus merguiensis*) were acquired from retail outlets. Prawns were derived from wild populations from different Australian States including Queensland, New South Wales and South Australia (Table 1). A single prawn from each location was preserved in 70% ethanol at 4^{o}C for confirmation of species identification.

Culturing and Initial Screening

Digestive tracts of four individual prawns from each location were each used to inoculate 5 ml of plate count medium (5 g tryptone, 2.5 g yeast extract, 1 g dextrose) and incubated at 25^{o}C overnight. The resulting mixed cultures were tested for the presence of *intI1*, *intI2* and *intI3* using boiled lysates (Stokes et al., 2006) with primer pairs HS915/HS916 (Gillings *et al.*, 2009), intI2F/intI2R and intI3F/intI3R (Mazel *et al.*, 2000) respectively. PCRs were performed in 50 µl reactions with GoTaq DNA polymerase (Promega, Madison, WI, USA) in the buffer supplied with the enzyme, 25 pmol of each primer and a final Mg concentration of 1.5 mM. PCRs were carried out using an Omn-E thermocycler (Hybaid, Middlesex, UK) with the following cycling programs: 94^{o}C for 3 min (one cycle); 94^{o}C for 30 s, 65^{o}C (*intI1*) or 60^{o}C (*intI2* and *intI3*) for 30 s, 72^{o}C for 90 s (35 cycles); 72^{o}C for 5 min (one cycle). PCRs were assessed using electrophoresis on 2% agarose gels cast in TBE buffer, post-stained in GelRed (Biotium) and visualized using UV transillumination (Yeates *et al.*, 1998).

Rescreening and Purification of *intI1* positive isolates

Mixed cultures that tested positive for *intI1* were serially diluted in 100 mM sodium phosphate buffer (pH 7.0) and 10^{-6} to 10^{-8} dilutions plated in triplicate onto PCA plates (5 g tryptone, 2.5 g yeast extract, 1 g dextrose and 12 g agar per liter). Plates were incubated at 25^{o}C for 2 days after which 100 individual colonies were picked into sterile microtiter trays containing 100 µl of PC broth. Trays were incubated overnight at 25^{o}C. DNA lysates were made from individual wells by harvesting 25 µl of broth in 25 µl of sterile water, heating at 99^{o}C for 10 min, snap chilling and centrifuging to pellet cell debris. The lysates were then used as a template for rescreening for *intI1* positive isolates by PCR as above.

Characterization of Cassette Arrays and Identification of MITEs

Microbial DNA was extracted from purified *intI1* positive bacterial isolates using a bead beating method (FastPrep^{TM} Bio101) (Yeates *et al.,* 1998). DNA from all *intI1* positive isolates was confirmed by PCR amplification of a 473-bp *intI1* gene fragment using primers HS463a and HS464 (Stokes *et al.*, 2006). Integrated cassette arrays were amplified using primers HS458 and HS459 (Stokes *et al.*, 2006; Hardwick *et al.*, 2008). The presence of MITEs in all isolates was tested using primers MRG310 (5' TTGTAGCAGTGCATTTGTTG) and MRG311 (5' AAACGGTGATTGCAAGTG). The resulting PCR products were assessed by 2% agarose gel electrophoresis as above.

Fosmid Library Construction

A fosmid library was previously constructed from the genomic DNA of isolate NFM2 (*A. johnsonii*) (Gillings *et al.*, 2009) using a CopyControl fosmid library production kit (Epicenter) as described by Stokes et al., (2006). The fosmid library of NFM2 was characterised by primer walking in either direction from the fosmid clone identified as containing the integron (Stokes *et al.*, 2006). This allowed us to explore the genomic landscape around the class 1 integron and design primers representing landmarks on either side of the integron (Table 2). These primer pairs were then used to investigate the genomic landscape around all *intI* positive isolates using PCR amplification. Maps of all isolates were generated and compared using these data.

Southern Hybridization

Genomic DNA extracted using bead beating (Yeates *et al.*, 1998) and plasmid DNA prepared as described by Kado and Liu (1981) were generated from each isolate. DNA samples were separated on 0.8% agarose and transferred to a nylon membrane (Amersham Biosciences) using standard protocols (Sambrook and Russell, 2001). The DNA was fixed to the membrane by UV cross linking (Stratalinker® UV Crosslinker 1800) at 1200

µjoulesx100 for 30 seconds. *IntI* digoxigenin (DIG)-labelled probes were prepared with the PCR DIG probe synthesis kit (Roche Diagnostics) using the *intI1* PCR conditions above. Overnight hybridization at 52.5°C was performed with *intI1* digoxigenin (DIG)-labelled probes followed by chemiluminescent detection of labelled DNA according to the DIG system user's guide (Roche Diagnostics).

Conjugation Experiments

Conjugation experiments were conducted by broth mating (Phornphisutthimas *et al.*, 2007) of *intI1* positive (donor) and *intI1* negative (recipient) strains. In these experiments the NFM2 donor (*A. johnsonii,* streptomycin resistant) was mated with one of three recipients including *E. coli DH5α* (nalidixic acid resistant), 12CE2 (*A. johnsonii*, Rifampicin resistant) and 12CE11 (*A. lwoffii,* Rifampicin resistant) (Table 1). Transconjugants from these experiments were selected on LB plates containing appropriate antibiotics, using 25 ug/ml of streptomycin and nalidixic acid and 50 ug/ml of rifampicin.

16SrDNA and 16S-23S IGS PCR

16S rDNA and 16S-23S intergenic spacer region amplifications were carried out for all *intI1* positive isolates. PCR amplification of 16S rDNA was carried out at 60^{o}C using primers f27 and r1492 (Weisburg *et al.*, 1991). 16S-23S IGS PCR was performed using primers 1512F and 6R (Chang *et al.*, 2005) with the following cycling conditions 1 cycle of 94^{o}C for 2 min; 35 cycles of 94^{o}C for 1 min, 62^{o}C for 1 min, 72^{o}C for 1 min; and 1 cycle of 72^{o}C for 7 min (Chang *et al.*, 2005). PCR products were sequenced directly after purification using Promega Wizard PCR columns. A subset of 16S-23S IGS PCR products were cloned to allow unamibiguous sequence reads.

Cloning and Plasmid Isolation

Purified PCR products were ligated into a TA cloning vector followed by transformation into TOP10 *E.coli* competent cells (Invitrogen) using a heat shock method as per the

manufacturer's protocol. Clones with inserts were identified by blue/white selection on LB agar plates containing 50 mg/ml of ampicillin, 24 mg/ml of IPTG (isopropyl beta-D-thiogalactopyranoside) and 40 mg/ml of XGal, (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). Ten white colonies were randomly picked from each sample and amplifications of inserts were carried out on boiled lysates from single colonies using the original PCR primers. Plasmids were then isolated from 5 ml overnight liquid cultures using the Wizard Miniprep DNA purification system (Promega) according to the manufacturer's instructions.

BOX and ERIC PCR

Genomic fingerprints of all *Acinetobacter* strains were generated using BOX and ERIC-PCR. PCR amplification was performed using Genereleaser^{TM} (Bioventures Inc.) to minimize PCR inhibitors as described by Gillings and Holley (1997). The following cycling programme was used: 1cycle of 94^{o}C for 3 min; 35 cycles of 94^{o}C for 30 s, 52^{o}C for 90 s, 68^{o}C for 8 min; and one cycle of 68^{o}C for 8 min (Gillings and Holley, 1997). Resultant PCR products were analysed on 2% agarose gels followed by staining and visualisation as described above.

Sequencing and Phylogenetic Analysis

Sequencing was performed at the Macquarie Sequencing Facility using dye terminator technology. Sequences were determined on an ABI 3130xl Genetic analyser using BigDye v 3.1 chemistry (Applied Biosystems). Phylogenetic analysis was conducted using bioinformatics software available through the Biomanager facility of ANGIS (http://www.angis.org.au/). BLASTN and BLASTX searches were performed through the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine nucleotide and protein homology in existing databases. 16S rDNA and 16S-23S IGS sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). Trees were constructed from both alignments

using MEGA4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the UPGMA method (Sneath, 1973), generating a bootstrap consensus tree from 500 replicates. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). All positions containing gaps were eliminated from the dataset . There were a total of 649 positions in the final dataset.

RESULTS

Over 75% of mixed cultures generated from the digestive tract of prawns tested positive for the class 1 integron-integrase gene (*intI1*). Positives were obtained from both species of prawn and from across the entire geographic range examined, covering Queensland, New South Wales and South Australia (Table 1). No positives were obtained for class 2 or class 3 integrase genes. Pure cultures were generated from the initial mixed cultures and rescreened for *intI1*. Twelve of about seven hundred pure cultures were positive for *intI1*.

All *intI* positive isolates were 16S rDNA sequenced, and these sequences compared with those available in GenBank. The 16SrDNA sequences of the *intI1* positive bacteria showed closest species matches with one of three *Acinetobacter* groups, *Acinetobacter johnsonii*, *Acinetobacter lwoffii* or an unnamed *Acinetobacter sp.* (Table 1, Fig. 1). We constructed a phylogenetic tree using the 16SrDNA sequences obtained from *Acinetobacter* strains recovered during this study. We included two *intI1* negative *Acinetobacter* strains also recovered from prawns (Table 1), and a panel of *Acinetobacter* isolates from clinical sources (Valenzuela *et al.*, 2007) that had also been DNA sequenced. Reference sequences of relevant *Acinetobacter* species were retrieved from Genbank to include in the phylogenetic analysis (Accession numbers are provided in Fig. 1). The phylogenetic tree confirmed that the *Acinetobacter* species recovered from prawns comprised three clades,

each with high levels of bootstrap support, and that these clades were distinct from *Acinetobacter* isolates from clinical contexts (Fig. 1). Analysis of the 16S-23S intergenic spacer sequences from all isolates was consistent with the conclusions drawn from 16S sequence data. Sequences generated during this work have been lodged with Genbank under Accession No.s **JX104145-JX104153**.

The class 1 integrons in all isolates recovered from prawns were mapped using a combination of PCR and DNA sequencing. All integrons had the same structure, and this was identical to that originally described from *Acinetobacter johnsonii* NFM2, also isolated from the digestive tract of a prawn (GenBank accession number FJ711439) (Gillings *et al.*, 2009). The order of elements in this structure was MITE – *intI1* – *attI1* – *msr* gene cassette – *aadA2* gene cassette 3'-CS – MITE (Fig. 3). Because mapping showed this identical structure was present in a number of *Acinetobacter* species, it was clearly moving between species by lateral gene transfer. Further, characterizing our collection of isolates using BOX and ERIC PCR demonstrated that there was considerable clonal diversity within the three *Acinetobacter* species groups we identified (Fig. 2). The presence of multiple clones was also confirmed by the microdiversity apparent in 16S sequences within species groups (Fig. 1). ERIC PCR showed a similar pattern of classification to that obtained by 16S rDNA analysis, defining three groups of *Acinetobacter* species. Consequently, it appeared that the integron was capable of frequent lateral transfer between clonal lineages and between species of *Acinetobacter.*

All prawn associated *intI1* positive *Acinetobacter* isolates tested positive for MITE elements using PCR with internal MITE primers (MRG310 and MRG311). To determine if the movement of the integron was being mediated by the flanking MITEs, we used PCR

across the boundaries of both proximal and distal MITEs with primer pairs 4G5-1F/HS463a and 4G5-tniA-F/4G5-1R respectively (Fig. 3, Table 2). All *intI1* positive *Acinetobacter* isolates from prawns generated PCR products of the expected size, demonstrating that the MITEs were flanked by the same DNA sequences in all three species. This, in turn, suggested that the integron was being mobilized by a larger DNA element into which the MITE – integron – MITE structure had become embedded. Consequently, we set out to determine if the integron was plasmid or chromosomally borne.

Three lines of evidence strongly suggested the integron was not on a plasmid. Firstly, Southern hybridization with *intI1* probes was carried out on genomic DNA (Yeates *et al.*, 1998) and on high molecular weight plasmid extractions (Kado and Liu, 1981) from environmental and clinical *Acinetobacters*. When *Acinetobacter* isolates from prawns were tested, hybridization of *intI1* gene probes only occurred to the chromosomal DNA. No hybridization was observed to any of the plasmid bands visible on agarose electrophoresis of these same isolates, suggesting that *intI* was localized on chromosomal DNA.

Secondly, mating essays were performed to conjugate the class 1 integron from the *intI1* positive donor NFM2 to various *intI1* negative recipients including *E. coli DH5α, Acinetobacter* isolates 12CE2, and 12CE11 (Table 1). However, all attempts remained unsuccessful, and did not identify any transconjugants on selection with appropriate antibiotics. This suggested that the class 1 integron was not associated with a readily conjugatable element (data not shown).

Thirdly, we extended our sequence data in either direction from the integron, using primer walking in the fosmid library originally generated from isolate NFM2 (Gillings *et al.*, 2009). That is, by using PCR based on the termini of the integron-containing fosmid, we identified those fosmids that contained the upstream and downstream regions, end sequenced these, designed new primers, and rescreened the library for fosmids representing the next flanking sequences. This process was continued for six cycles towards left hand side and three cycles towards right hand side. All sequence data generated were used to interrogate DNA databases. The majority of sequences showed significant matches with conserved hypothetical proteins, or had no homologues in the database (data not shown). No evidence of plasmid associated sequences was found. The fosmid walking procedure gave us landmarks (here labelled for convenience A-N, Fig. 3) to investigate the genomic context of the class 1 integrons in all the *Acinetobacter* isolates from prawns. The primers designed for fosmid walking were used in PCR to assess the presence of genomic regions on either side of the integron. Schematic maps were then constructed and compared for all the isolates examined in this paper (Fig. 3). As expected, the *A. johnsonii* isolate NFM2, from which the fosmid library was originally constructed, was positive for all PCRs. Two further *intI1* positive *A. johnsonii* isolates from prawn (1DG10 & 10CB3) gave identical results to isolate NFM2. One isolate of *A. johnsonii* recovered from a prawn (11BF2) was positive for landmarks H,I and J which represents integron/MITE structure, while testing negative for all flanking PCRs (landmarks, A to G and K to N).

One isolate of *A. johnsonii* recovered from a prawn (12CE2) was negative for *intI1* and also tested negative for all genomic landmarks A through N (Fig. 3). This suggested that there were three distinct arrangements with respect to the class 1 integron in our isolates of

A. johnsonii. The first (1DG10 & 10CB3) carried the class 1 integron/MITE structure flanked by a long genomic segment, and was identical to the previously described isolate NFM2. The second (11BF2) contained only the integron/MITE without the same extensive flanking sequence, and the third (12CE2), contained neither the flanking region nor the integron/MITE element.

PCR mapping of the *A. lwoffii* isolates gave similar results. Two *intI1* positive isolates (12CE1 and 12 CE12) tested positive in PCRs for all genomic landmarks A to N (Fig. 3). The integron negative isolate 12CE11, also *A. lwoffii,* was negative for all PCRs including those targeting the class 1 integron. Comparing the ERIC PCR profiles of 12CE12 (*intI1* positive) and 12 CE11 (*intI1* negative) showd that they were very closely related, if not clonal, differing only by the presence of an additional band of approximately 500 bp in isolate 12CE12. The simplest conclusion is that the extra PCR product was generated from the region that contains the integron in isolate 12CE12. This 500 bp ERIC-PCR band is seen in all the other integron positive environmental *Acinetobacter spp.* described in this paper (Fig. 2), lending weight to this conclusion.

The final group of isolates from prawns all belonged to an unnamed *Acinetobacter* species. All seven isolates were *intI1* positive and positive in PCRs for all landmarks A through N (Fig. 3). The simplest explanation for the combined results from all three species of *Acinetobacter* is that a segment of DNA that carries the MITE-associated class 1 integron was moving between clones and species of *Acinetobacter* that colonise the gut of prawns.

We also tested a panel of *Acinetobacter baumanii* and other strains isolated from clinical settings (Valenzuela *et al.*, 2007) for the presence of the PCR landmarks. Although the

majority of these isolates carried class 1 integrons, these did not have flanking MITE elements, and were more typical of plasmid borne integrons. The majority of isolates were also negative for all PCR landmarks. However, one isolate belonging to *Acinetobacter nosocomialis* (WM98b) (Valenzuela *et al.*, 2007; Nemec *et al.*, 2011) tested positive for landmarks A through G and K through N (Fig. 3), but was not positive for MITE elements nor the associated class 1 integron. Consequently, the genomic segment subject to transfer in the prawn isolates was also present in this clinical *Acinetobacter*, but it did not carry the class 1 integron/MITE element. A hypothetical model for the dissemination of the *intI*/MITE element and its flanking genomic segment among various strains of *Acinetobacter* is shown in Fig. 4.

DISCUSSION

In this study we surveyed bacteria isolated from the digestive flora of wild prawns for the presence of class 1 integrons. We recovered 12 isolates which were positive for the same unusual integron previously described from a single prawn (Gillings *et al.*, 2009). This unusual integron was recovered from prawns collected across a wide geographic range, covering some 2,500 km, and was present in three species, identified using 16S sequence analysis as *Acinetobacter johnsonii, A. lwoffii* and a further unnamed *Acinetobacter* species.

The integron possessed features typical of Tn*402*-derived class 1 integrons, including the presence of a 3'-CS and a truncated *tni* module. This strongly implies a clinical origin for this element, as does the fact that one of the cassettes present in the array, *aadA2*, is extensively dispersed amongst class 1 integrons of clinical origin (Partridge *et al.*, 2009). During its subsequent release into the environment, this element has been modified such

that it became flanked by miniature inverted repeat transposable elements (MITEs) that replaced the terminal inverted repeats (IRi and IRt) of more typical Tn*402* transposons (Gillings *et al.*, 2009). The class 1 integron now linked to these MITEs has also captured an unusual gene cassette (*msr*), encoding potential pathogenicity factors. Since this cassette has not previously been detected in class 1 integrons from clinical sources, we infer that this capture event occurred after release of the integron back into the environment. Here we have shown that this unusual class 1 integron is being actively exchanged between the members of genus *Acinetobacter* in a marine ecosystem.

PCR mapping of the flanking regions surrounding the class 1 integron demonstrated that the integron was probably part of a much larger DNA segment that was being mobilized between diverse *Acinetobacter* strains and species. This same segment was detected in an opportunistic pathogen, *A. nosocomialis*, but in this case the integrin/MITE region was not present. Large genomic islands containing integrons, multiple resistance loci, metal resistance genes and transposition machinery are being increasingly reported from *Acinetobacter* isolated in clinical contexts (Fournier *et al.*, 2006; Krizova and Nemec, 2010; Post *et al.*, 2010; Krizova *et al.*, 2011). However, the element we have partially characterized here did not show homology to any previously described genomic island, or exhibit sequence signatures attributable to any described islands.

Bacteria belonging to the genus *Acinetobacter* are generally regarded as ubiquitous microorganisms, since they are found frequently in soil, water and hospital environments (Towner, 1996; Peleg *et al.*, 2008). Previous studies have shown that *Acinetobacter lwoffii* and *Acinetobacter johnsonii* are predominantly isolated from food, human skin, and clinical contexts (Gennari and Lombardi, 1993; Seifert *et al.*, 1994; Seifert *et al.*, 1997)

and therefore, appear to possess a high degree of adaptability to various ecological niches. In addition, these species could play an important role in potential transfer of genetic information between clinical and natural environments. A recent study conducted in Portugal (Domingues *et al.*, 2011) identified a class 1 integron flanked by MITEs from a clinical *A. baumannii* strain, isolated from a human urine sample. The MITEs, *intI1* and 3'CS were identical to the sequences we have described here, but the gene cassette content was different, and the entire structure was inserted into a different genetic context. This observation suggests the potential for MITE mediated translocation of class 1 integrons among various strains of *Acinetobacter*.

The likely evolution of the unusual class 1 integron described in this paper, and its subsequent dissemination can at least be partially reconstructed (Fig. 4). A typical *Tn*402-like class 1 integron containing a 3'-CS and a cassette array containing an *aadA2* gene cassette was released into the general environment from an anthropogenic source. Here it acquired the *msr* gene cassette, and the terminal repeats were replaced by MITE elements. This compound element was subsequently captured by a genomic island, and this genomic island was then extensively transferred between various strains and species of *Acinetobacter* resident in the digestive system of prawns. The presence of the *msr* genes, which encode methionine sulfoxide reductase, involved in the repair of protein damage during oxidative stress (Grimaud *et al.*, 2001), potentially enhances bacterial colonization and pathogenicity.

We have detected the same genomic island in a strain of *A. nosocomialis* isolated from a clinical context, but without the embedded integron. Further, the MITE-integron-MITE element has been detected in *A. baumannii*, but without the associated genomic island

(Domingues *et al.*, 2011). Clearly there is potential for this genomic island to transfer into clinically important *Acinetobacter* strains, especially given its residence in the digestive system of a popular food species that is only lightly cooked before consumption. The element we have described here adds another weapon to the potential evolutionary arsenal of this formidable opportunistic pathogen.

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FIGURE LEGENDS

Figure 1: Phylogenetic tree of *Acinetobacter* isolates used in this study. The tree is based on partial 16S rDNA sequences of *Acinetobacter* strains isolated from prawn digestive systems and a panel of *Acinetobacter* isolates from clinical contexts (Valenzuela *et al.*, 2007). The tree also includes reference *Acinetobacter* species retrieved from GenBank, whose accession numbers are shown in brackets. Prawn-associated *Acinetobacter* strains grouped into three clades: *A. johnsonii, A. lwoffii*, and an unnamed *Acinetobacter* species and shown in bold. Clinical isolates included *A. baumannii*, *A. pittii* and *A. nosocomialis*. Bootstrap values greater than 80% are shown. The horizontal scale shows rate of substitutions per nucleotide.

Figure 2: Clonal variation amongst *Acinetobacter* species recovered from prawns. DNA fingerprints were generated using ERIC-PCR (Gillings and Holley, 1997). Diversity is evident both within and between isolates of each species. Two *intI1* negative isolates (12CE11 and 12CE2) do not exhibit the strong 500 bp band seen in all *intI1* positive isolates (arrow). The far left hand lane contains a 100 bp ladder, used as a size marker.

Figure 3: PCR mapping of all *Acinetobacter* strains. The structure of the class 1 integron from prawns is shown, consisting of: a miniature inverted repeat transposable element (MITE); the class 1 integron-integrase gene (*intI1*); a gene cassette containing *msrB* and *msrA* genes plus a hypothetical open reading frame (*ctr*); a gene cassette containing *aadA2*; a typical 3'-CS; and a second MITE. The primer pairs used for detecting the presence of this integron in bacterial isolates are shown under the map. These three PCR assays correspond to landmarks H, I and J in the lower part of the figure. The entire region G

through K was DNA sequenced in isolate NFM2 (Accession number FJ711439), and the terminal sequences used to design PCR assays to recover overlapping fosmids in the original fosmid library generated from NFM2 (see (Gillings *et al.*, 2009). Termini of these fosmids were sequenced and the PCR-primer walking process repeated for six cycles on the right hand side and three on the right, generating landmarks A through F, and L through M respectively. The presence of these landmarks, as assessed by PCR assay, are shown as filled boxes for each of the isolates from prawns, and for the one clinical isolate (*A. nosocomialis* WM98b) that tested positive in the PCRs. Isolates 12CE2 and 12CE11 were negative for *intI1* and all flanking PCRs, represented by horizontal broken lines. One isolate (11BF2) was positive for the *intI1*/MITE region and tested negative for all flanking PCRs.

Figure4: Hypothetical model for the origin and dissemination of the *intI1*/MITE element commonly found in prawns. (A) A class 1 integron, typical of those found in clinical contexts, is released into the environment from a human dominated ecosystem. (B), (C) The structure is modified by insertion of the *msr* gene cassette, and by replacement of the terminal repeats (IRi and IRt) with MITEs to generate (D), a MITE associated class 1 integron, such as that detected in *Acinetobacter johnsonii* 11BF2 (Figure 3). (E) The MITE-associated integron is captured by a genomic island, such as that detected in *A. nosocomialis* WM98b (Figure 3). (F) The genomic island with embedded integron is disseminated between species and strains of *Acinetobacter*. It seems likely that many of the events described occurred within *Acinetobacter*, resident within the prawn gut, but this cannot be known with any certainty.

TABLE 1 *Acinetobacter* strains used in this study

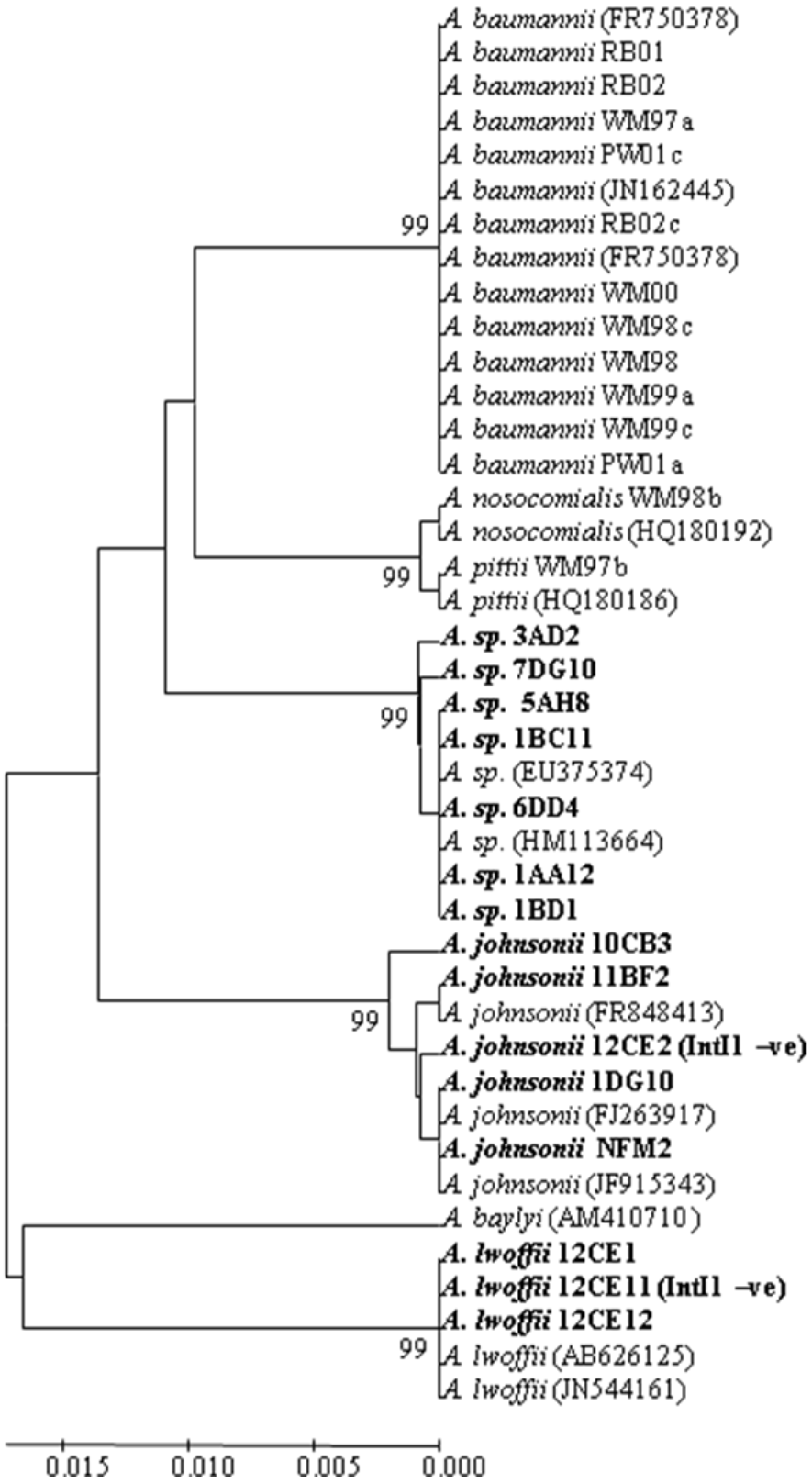
Culture ID	Species	Source	Location	*intI1* PCR[a]	Cassette Array[b]	MITE PCR[c]
1AA12	*Acinetobacter sp.*	King Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
1BC11	*Acinetobacter sp.*	King Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
1BD1	*Acinetobacter sp.*	King Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
3AD2	*Acinetobacter sp.*	Banana Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
5AH8	*Acinetobacter sp.*	King Prawn	South Australia	+	*msrB-msrA*-ctr|*aadA2*	+
6DD4	*Acinetobacter sp.*	King Prawn	Queensland	+	*msrB-msrA*-ctr|*aadA2*	+
7DG10	*Acinetobacter sp.*	Banana Prawn	Queensland	+	*msrB-msrA*-ctr|*aadA2*	+
1DG10	*Acinetobacter johnsonii*	King Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
10CB3	*Acinetobacter johnsonii*	Banana Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
11BF2	*Acinetobacter johnsonii*	King Prawn	NSW	+	*msrB-msrA*-ctr|*aadA2*	+
NFM2	*Acinetobacter johnsonii*	King Prawn	Australian east coast	+	*msrB-msrA*-ctr|*aadA2*	+
12CE2	*Acinetobacter johnsonii*	King Prawn	Queensland	-	-	-
12CE1	*Acinetobacter lwoffii*	King Prawn	Queensland	+	*msrB-msrA*-ctr|*aadA2*	+
12CE11	*Acinetobacter lwoffii*	King Prawn	Queensland	-	-	-
12CE12	*Acinetobacter lwoffii*	King Prawn	Queensland	+	*msrB-msrA*-ctr|*aadA2*	+
WM98	*Acinetobacter baumannii*	Wound	Westmead Hospital, NSW	+	*aacC1|orfP|orfP|orfQ|aadA1*	-
WM97a	*Acinetobacter baumannii*	Sputum	Westmead Hospital, NSW	+	*dfrA17|aadA5*	-
WM97b	*Acinetobacter pittii*	Not recorded	Westmead Hospital, NSW	+	-	-
WM98b	*Acinetobacter nosocomialis*	Sputum	Westmead Hospital, NSW	+	*dfrA17|aadA5*	-
WM98c	*Acinetobacter baumannii*	Not recorded	Westmead Hospital, NSW	+	-	-
WM99a	*Acinetobacter baumannii*	Not recorded	Westmead Hospital, NSW	+	-	-
WM99c	*Acinetobacter baumannii*	Sputum	Westmead Hospital, NSW	+	*aacC1|orfP|orfP|orfQ|aadA1*	-
WM00	*Acinetobacter baumannii*	Bronchoalveolar lavage	Westmead Hospital, NSW	-	-	-
RB02	*Acinetobacter baumannii*	Rectal swab	Royal Brisbane Hospital, Queensland	+	*aacC1|orfP|orfP|orfQ|aadA1*	-
RB02c	*Acinetobacter baumannii*	Rectal swab	Royal Brisbane Hospital, Queensland	-	-	-
RB01	*Acinetobacter baumannii*	Blood culture	Royal Brisbane Hospital, Queensland	+	*aacC1|orfP|orfP|orfQ|aadA1*	-
PW01a	*Acinetobacter baumannii*	Not recorded	Prince of Wales Hospital, NSW	+	*aacC1|orfP|orfP|orfQ|aadA1*	-
PW01c	*Acinetobacter baumannii*	Not recorded	Prince of Wales Hospital, NSW	+	*aacC1|orfP|orfP|orfQ|aadA1*	-

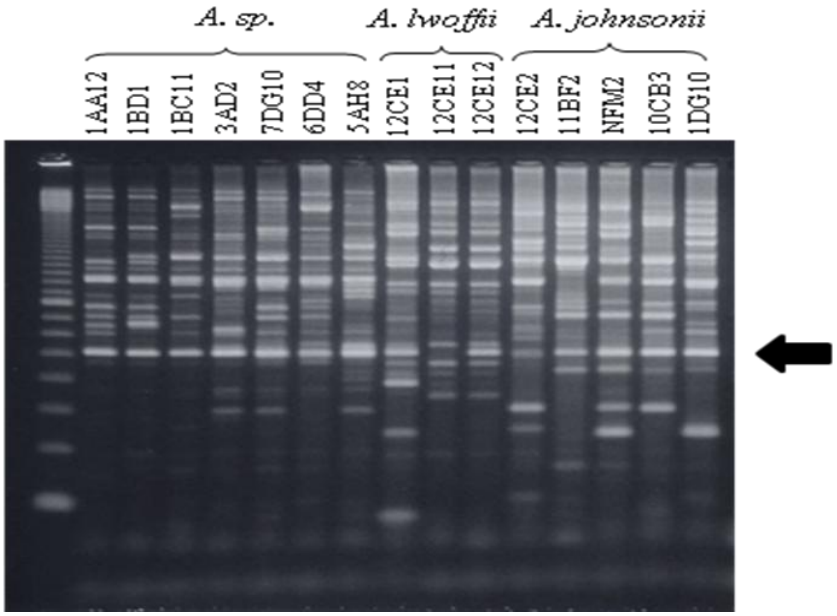
[a] Isolate tested positive for *intI1* using primer pairs HS915/HS916 (Gillings *et al.*, 2009) and HS463a/HS464 (Stokes *et al.*, 2006); [b] Cassette array identified using PCR and DNA sequencing for prawn isolates, information from (Valenzuela *et al.*, 2007) for clinical isolates; [c] The presence of MITEs in all isolates was tested using primer pair MRG310 and MRG311.

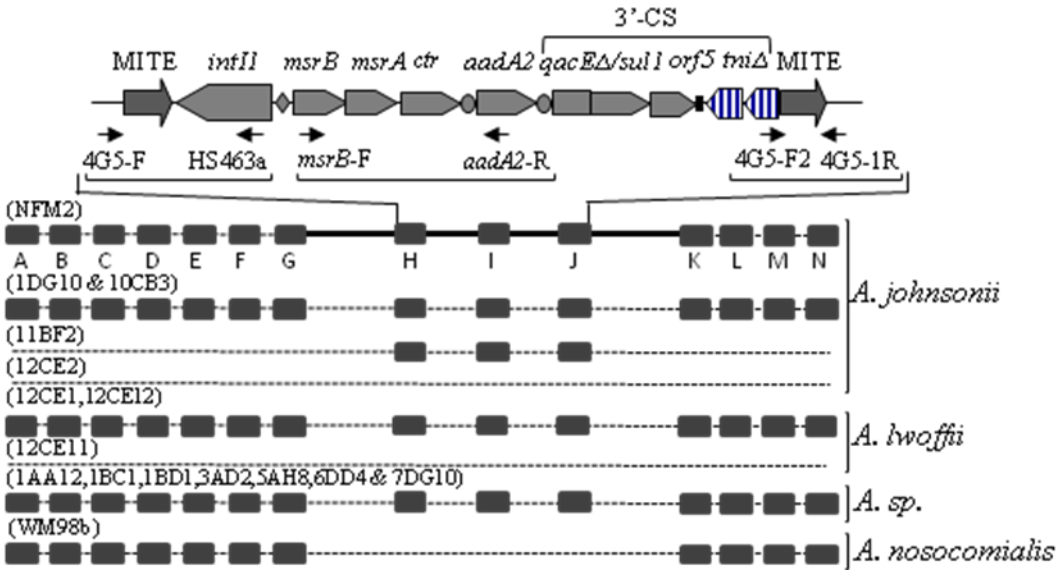
TABLE 2 Primers used for fosmid walking and PCR screening of *Acinetobacter* strains

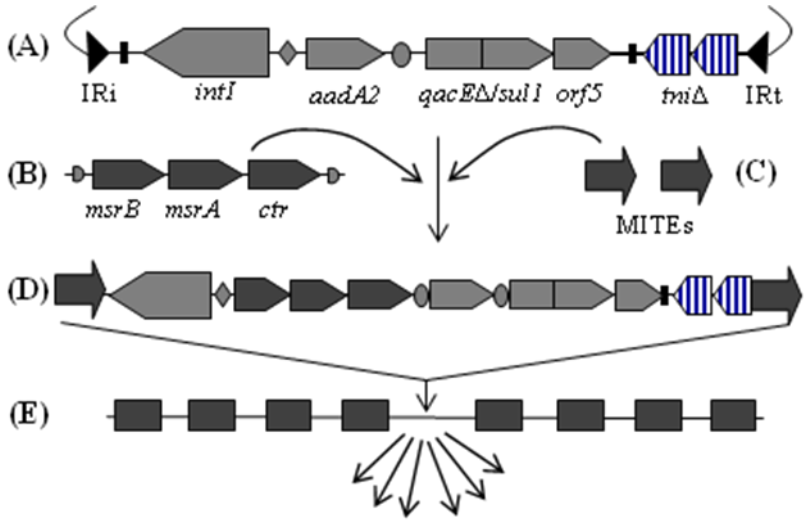
Landmarks[a]	Primer pairs	Sequence (5' to 3')	Reference
Landmark A	MRG376	GTGCTTTCACACACGAGATC	This study
	MRG377	CGCGACAAAGTTGATGTTAC	This study
Landmark B	MRG378	GCCGTGGTATCACCTACTTG	This study
	MRG379	CCGATGTTTGGTCGTAAACC	This study
Landmark C	MRG326	TTCAGCGGTGAACAGATTCC	This study
	MRG327	GCCGTTGGATCGATGTATTG	This study
Landmark D	MRG374	ACGGCGATCTGCTCTAAC	This study
	MRG375	GGCCAGACAGCAATCATG	This study
Landmark E	MRG312	GTTATACGCTGGCCAGACTGC	This study
	MRG313	TGACTTTCCGCACGAACG	This study
Landmark F	MRG324	TCCGAGATGGACGCTCTG	This study
	MRG325	GATCGGCACACGACAACC	This study
Landmark G	MRG308	GACCACCCTTATGGTTTAGTGC	This study
	MRG309	TCACCACTACCACCCTTAATCC	This study
Landmark H	4G5-F	CCCACACAATAAACGCCG	Gillings *et al*., 2009
	HS463a	CTGGATTTCGATCACGGCACG	Stokes *et al*., 2006
Landmark I	msrB-F	TGGAAGCGGAAGGCTACG	This study
	aadA2-R	AGATGGCGCTCAATGACG	This study
Landmark J	4G5-1R	TGGCGATGGC TCAATGTC	Gillings *et al*., 2009
	4G5-F2	TGCGACAAGGTACGGTAGG	Gillings *et al*., 2009
Landmark K	MRG306	ATGATGTTAGACGGCGTTCC	This study
	MRG307	TGTGGACGGATGTTTACACG	This study
Landmark L	MRG330	ATGTGGAAGCTTGGGCTTTG	This study
	MRG331	CTTGATAAAGCGCTGGAGCTG	This study
Landmark M	MRG314	GCCAAGGCCCAAATCATCC	This study
	MRG315	GTCGGCAAGGTGGGTGTTG	This study
Landmark N	MRG328	AGCCACTGAATGAGTCGTTG	This study
	MRG329	TCAGGGTAGAGCTGAGTATAGGG	This study

[a] Landmarks are detailed in Fig. 3, and represent map positions derived from overlapping fosmids generated from *A. johnsonnii* strain NFM2. Landmarks H to J cover the class 1 integron and its flanking MITE elements.


A. baumannii (FR750378)
A. baumannii RB01
A. baumannii RB02
A. baumannii WM97a
A. baumannii PW01c
A. baumannii (JN162445)
99
A. baumannii RB02c
A. baumannii (FR750378)
A. baumannii WM00
A. baumannii WM98c
A. baumannii WM98
A. baumannii WM99a
A. baumannii WM99c
A. baumannii PW01a
A. nosocomialis WM98b
A. nosocomialis (HQ180192)
99
A. pittii WM97b
A. pittii (HQ180186)
A. sp. 3AD2
A. sp. 7DG10
99
A. sp. 5AH8
A. sp. 1BC11
A. sp. (EU375374)
A. sp. 6DD4
A. sp. (HM113664)
A. sp. 1AA12
A. sp. 1BD1
A. johnsonii 10CB3
A. johnsonii 11BF2
99
A. johnsonii (FR848413)
A. johnsonii 12CE2 (IntI1 –ve)
A. johnsonii 1DG10
A. johnsonii (FJ263917)
A. johnsonii NFM2
A. johnsonii (JF915343)
A. baylyi (AM410710)
A. lwoffii 12CE1
A. lwoffii 12CE11 (IntI1 –ve)
A. lwoffii 12CE12
99
A. lwoffii (AB626125)
A. lwoffii (JN544161)
0.015
0.010
0.005
0.000


A. sp.
A. lwoffii
A. johnsonii
1AA12
1BD1
1BC11
3AD2
7DG10
6DD4
5AH8
12CE1
12CE11
12CE12
12CE2
11BF2
NFM2
10CB3
1DG10


3'-CS
MITE
intI1
msrB
msrA
ctr
aadA2
qacEΔ/sul1
orf5
tniΔ
MITE
4G5-F
HS463a
msrB-F
aadA2-R
4G5-F2
4G5-1R
(NFM2)
A B C D E F G H I J K L M N
(1DG10 & 10CB3)
(11BF2)
(12CE2)
(12CE1,12CE12)
(12CE11)
(1AA12,1BC1,1BD1,3AD2,5AH8,6DD4 & 7DG10)
(WM98b)
A. johnsonii
A. lwoffii
A. sp.
A. nosocomialis



(F) Dissemination of GI/IntI1/MITE into *Acinetobacter* strains and species

CHAPTER 6 - FOSMID LIBRARY CONSTRUCTION AND SCREENING

6.1 - Introduction

Integrons are genetic elements that possess a site specific recombination system that is able to capture and express diverse gene cassettes, and thus contribute to lateral gene transfer in bacteria. Integrons may be embedded within bacterial chromosomes or found as part of various mobile elements, such as transposons and plasmids (Rowe-Magnus *et al.*, 1999; Stokes *et al.*, 2006; Boucher *et al.*, 2007). Most integrons from environmental bacteria are inserted into chromosomes, and are confined to particular bacterial lineages. In contrast, clinically important integrons (e.g. class 1 integrons) are generally embedded in mobile elements which facilitate their lateral transfer into a wide range of pathogens (Stokes *et al.*, 2006; Gillings *et al.*, 2008a). It now seems clear that chromosomal integrons are the ancestral form of mobilized integrons such as the class 1 integron (Rowe-Magnus *et al.*, 2001; Rowe-Magnus *et al.*, 2002; Mazel, 2006; Gillings *et al.*, 2008a).

The most common method to determine if integrons are on either a plasmid or a chromosome is to characterize the regions located downstream and upstream of the integron. In chromosomal integrons, diverse arrays of genes have been identified outside the integron region (Drouin *et al.*, 2002; Rowe-Magnus *et al.*, 2003; Gillings *et al.*, 2005). Often these encode conserved hypothetical proteins (Vaisvila *et al.*, 2001) or enzymes of central metabolic pathways (Rowe-Magnus *et al.*, 2003; Gillings *et al.*, 2005). In general, chromosomal integrons can be identified through having extensive flanking regions, with a preponderance of genes usually found chromosomally, and a lack of genes more typical of

plasmids and transposons. In contrast, mobile integrons are often flanked by genes encoding transposition functions, plasmid maintenance functions, or typical loci found on a broad range of mobile elements (Sundstrom *et al.*, 1991; Radstrom *et al.*, 1994; Kholodii *et al.*, 1995; Brown *et al.*, 1996).

This chapter describes a genomic library constructed from a strain of *Acinetobacter johnsonii* (isolate NFM2) that carried an unusual class 1 integron. The library was screened for fosmids containing the class 1 integron of interest, and flanking fosmids mapped by primer walking to determine if the integron was plasmid borne or chromosomal.

6.2 - Materials and Methods

6.2.1 - Fosmid Library Construction

High molecular weight genomic DNA was prepared as described in Chapter 2. The quality and size of sheared genomic DNA was assessed by 1% gel electrophoresis. A fosmid library from strain NFM2 (*Acinetobacter johnsonii*) was constructed using this DNA according to the manufacturer's directions (CopyControl^{TM} fosmid library production kit; Epicentre).

6.2.2 - Selection of Clones

DNA fragments were ligated into the fosmid vector followed by packaging into the lambda phage capsids, which were then used to transfect *Escherichia coli* as described in the Copy Control kit manual. Selection of clones was carried out on LB-chloramphenicol plates. After overnight incubation at 37 ^{0}C, 480 clones were selected for further analysis using methods previously described (Stokes *et al.*, 2006). All clones from this library were stored

at -80 ^{0}C in five 100-well plates in LB containing 12.5 ug/ml chloramphenicol and 15% glycerol.

6.2.3 - Screening and Purification of *intI1* Positive Clones

To screen the fosmid library for *intI1*, 15 µl were sampled from each storage well and pooled by row in Eppendorf tubes (180 µl per tube). Tubes were centrifuged, and cells were resuspended in 25 µl of pH 7.0 Tris-EDTA buffer. Two microliters of this suspension were added as template to PCRs using primers HS463a and HS464, to screen for the presence of *intI1*. If a positive result was obtained, each well of the positive row of the block was then screened individually. The resulting positive clones were re-streaked on LB agar plates containing 12.5 µg/ml of chloramphenicol and were then used to inoculate liquid LB culture for extraction of pure fosmid DNA (according to the CopyControl kit manual). The yield of purified fosmids was assessed using gel electrophoresis, visualized via staining with GelRed.

6.2.4 - PCR Recovery of Additional Integron Boundary Regions

The *intI1* positive fosmid clones were used to determine the complete sequence of the integron. This sequence was used to identify flanking upstream and downstream regions by primer walking. Primers were designed to the termini of the *intI1* positive fosmid clones, and these primers were then used for PCR screening of the fosmid library as described above. This protocol recovered fosmids representing the next flanking region upstream and downstream of the original fosmid. The resulting positive clones were purified and end sequenced to design new primers, which in turn were used to recover the next set of upstream and downstream fosmids. This procedure was repeated several times to extend the sequence outwards from the integron (Figure 6.1).

6.2.5 - Sequencing of Fosmid Clones

After purification, fosmids were end-sequenced using vector primers pCC2FOSF (5'GTACAACGACACCTAGAC 3') and pCC2FOSR (5'CAGGAAACAGCCTAGGAA3'). The sequences obtained were tested for nucleotide and protein homology in existing databases using BLASTN and BLASTX searches through the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.2.6 - PCR Screening of all *Acinetobacter* Strains by Using Primers Designed from the NFM2 Fosmid Library

All *Acinetobacter* strains recovered from prawn gut during this study (Chapter 5, Table 5.1), and a collection of other *Acinetobacter* isolates from clinical contexts (Chapter 5, Table 5.1), were PCR screened for the presence of the class 1 integron and for the flanking regions represented by the termini of the fosmids obtained during the primer walking process.

6.3 - RESULTS

6.3.1 - Recovery of the Complete Integron and Flanking Sequence

A. johnsonii isolate NFM2 was selected for construction of a fosmid library, with the aim of fully characterizing the class 1 integron known to be resident in this strain, and identifying the sequences flanking the integron. On screening with class 1 integron primers, three of the fosmid clones prepared from NFM2 (4G5, 5B9 and 4B11) tested positive. The integron region was completely sequenced using a combination of primers (Figure 6.1, Chapter 4). Additionally, fosmid 4G5 was completely sequenced using pyrosequencing (Ramaciotti Centre, University of NSW, Sydney, Australia). The sequence

of fosmid 4G5 was deposited in GenBank under accession number FJ711439, and is described in Gillings *et al.* (2009a), also included as Chapter 4 of this thesis. Sequence analysis revealed an unusual class 1 integron structure consisting of MITE-*intI1*-*attI1*-gene cassettes (*msrB*-*msrA*-*ctr* and *aadA2*)-*qacE*Δ*sul1*-*orf5*-*tniB*Δ*tniA*Δ-MITE (Figure 6.1) (Gillings *et al.*, 2009).

6.3.2 - PCR Recovery of Additional Integron Boundary Regions

We extended our sequence data in both directions from the integron by using the terminal sequences of the *intI1* positive fosmid clones 4G5 and 4B11 to design PCR primers. These primers were used to rescreen the fosmid library for clones representing the next flanking regions, and these positive clones were then purified and sequenced.

6.3.3 - Recovery of Left Hand Side (5') Boundary Region

Primers MRG308 and MRG309 were designed to the left terminus of the *intI1* positive fosmid clone 4G5 and used to screen the library for fosmids representing next flanking sequences. On PCR screening, fosmid clone 5E8 tested positive for MRG308/MRG309, but negative for *intI 1*. Consequently it represented the genomic region adjacent to the integron. This fosmid was purified and end sequenced. These end sequences were then used to design a second round of screening primers (MRG312 and MRG313) and the fosmid library tested to recover the next flanking region. This process was continued for another five cycles on the left hand side of the integron boundary. All primer locations and fosmids recovered during upstream primer walking are shown in Figure 6.1.

6.3.4 - Recovery of Right Hand Side (3') Boundary Region

Primers MRG306 and MRG307 were designed to the terminal sequence of fosmid clone 4B11. On PCR screening of fosmid library with this primer pair, two fosmids (2H7 and 3E12) tested positive. These fosmids represented the region flanking the right hand boundary of the integron (Figure 6.1). These fosmids were purified and sequenced to extend the right hand flanking sequence by primer design and screening as described above. This represents the first cycle of sequence extension from the right terminus of the integron and was continued for another two cycles. All fosmids identified during downstream primer walking are shown in Figure 6.1.

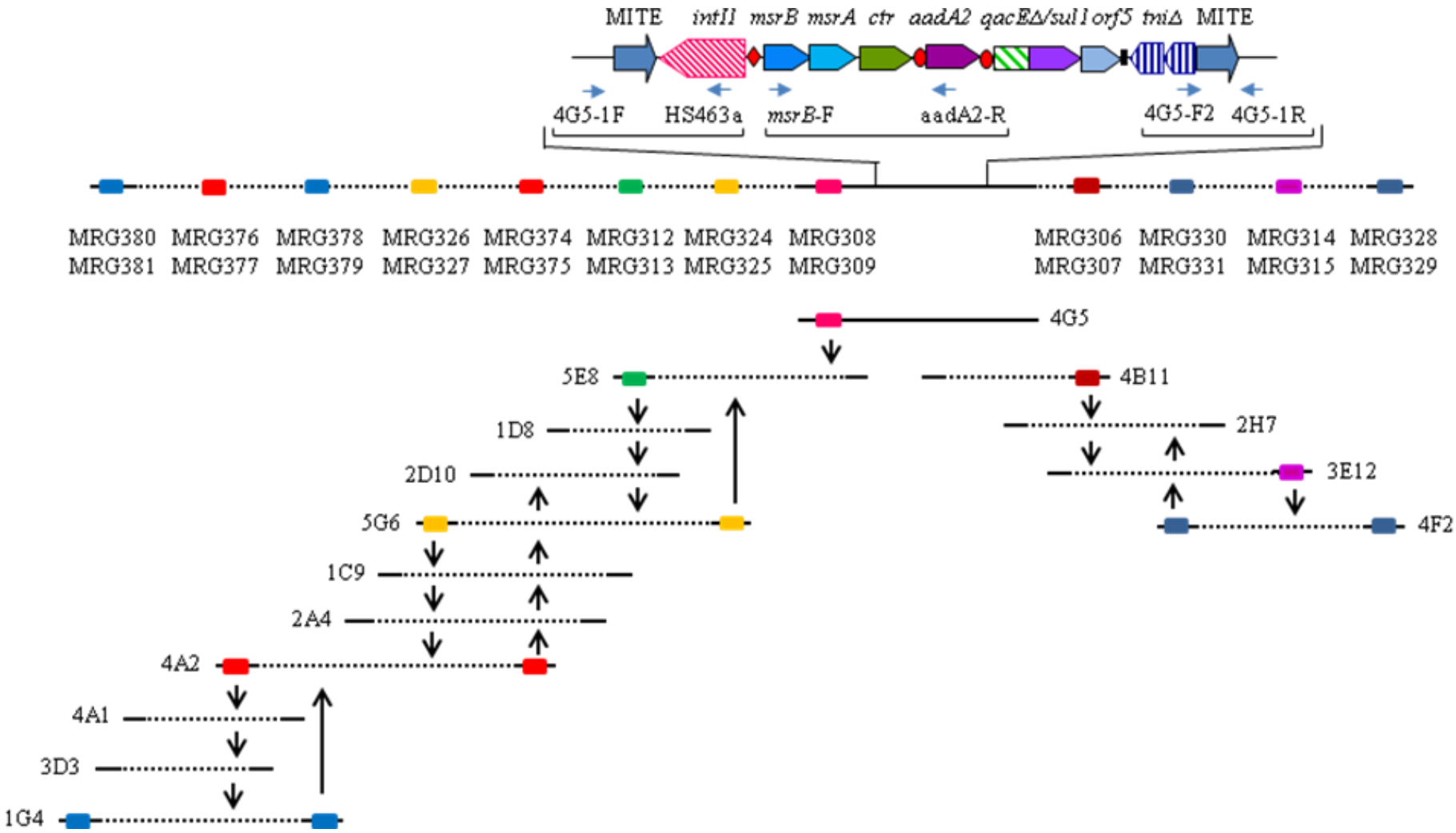

MITE
intI1
msrB
msrA
ctr
aadA2
qacEΔ/sul1
orf5
tniΔ
MITE
4G5-1F
HS463a
msrB-F
aadA2-R
4G5-F2
4G5-1R
MRG380 MRG376 MRG378 MRG326 MRG374 MRG312 MRG324 MRG308
MRG381 MRG377 MRG379 MRG327 MRG375 MRG313 MRG325 MRG309
MRG306 MRG330 MRG314 MRG328
MRG307 MRG331 MRG315 MRG329
4G5
5E8
4B11
1D8
2H7
2D10
3E12
5G6
4F2
1C9
2A4
4A2
4A1
3D3
1G4

Fig. 6.1 PCR mapping of regions flanking the class 1 integron in *Acinetobacter johnsonii* strain NFM2. The structure of the class 1 integron is shown, consisting of: a miniature inverted repeat transposable element (MITE); the class 1 integron-integrase gene (*intI1*); gene cassettes containing *msrB-msrA-ctr* and aadA2; *qacE*Δ*sul1*; *orf5; tniB*Δ*tniA*Δ and a second MITE . The primer pairs used for detecting the presence of this integron in the collection of *Acinetobacter* isolates from wild prawns are shown under the map.

To investigate the genomic context of the class 1 integron, the terminal sequences of fosmids 4G5 and 4B11 were used to design primers. PCR using these primers was then used to screen the NFM2 fosmid library to recover regions flanking the integron. This procedure recovered fosmid 5E8 on the left hand side, and fosmids 2H7 and 3E12 on the right hand side. The termini of these fosmids were sequenced and the PCR-primer walking process repeated. Six cycles of PCR screening were conducted for the left hand side and three for the right respectively. Fosmids identified during the walking process are shown in the Figure as a schematic map. Positive PCR results are shown by the vertical arrows, and these PCR results allowed fosmids to be oriented with respect to each other. Solid lines represent sections of fosmids that were DNA sequenced, and the colored boxes denote regions amplified in diagnostic PCR assays. Primer pairs used in these assays are noted below the upper schematic. A summary of the sequence data generated from the termini of each fosmid is given in Table 6.2.

6.3.5 - Sequence Analysis

Terminal fosmid sequences (sized between 800-1000 bp) were analysed and used to investigate the genomic landscape of the class 1 integron, particularly to determine if it were plasmid or chromosomally borne. The majority of sequences only showed significant matches to conserved hypothetical proteins, or had no significant homologies in the database (Table 6.1). The few sequences that showed significant homology to genes of known function encoded functional proteins such as type IV secretion proteins, transglutaminase-like domain proteins, sulphite reductase and putative transport proteins (Table 6.1). No evidence of plasmid-associated genes was found on sequence analysis.

6.3.6 - Screening of *Acinetobacter* Strains for Integron and Flanking Genes by Using NFM2 Fosmid Primers

The primers designed for the NFM2 fosmid walking procedure were used to map the integron and flanking regions from all *Acinetobacter* strains in our collection from prawns. This collection included two *intI1* negative (12CE2 & 12CE11) isolates from prawns and a collection of clinical *Acinetobacter* isolates known to be *intI1* positive. Schematic maps were constructed based on the results of the PCR assays and compared for all isolates in the collection (Figure 6.1, Chapter 5).

On PCR mapping, *intI1* positive *Acinetobacter* strains recovered from prawn gut (*Acinetobacter johnsonii, Acinetobacter lwoffii* and *Acinetobacter sp.*) tested positive in all PCRs designed for primer walking in the NFM2 fosmid library. The two *intI1* negative *Acinetobacter* strains tested negative for all PCRs. Of the clinical isolates, only one strain, *Acinetobacter nosocomialis* (WM98b) generated PCR products with primers designed from NFM2 sequence, whereas the remaining clinical isolates tested negative for all PCRs.

Table 6.1 BLAST results for the terminal sequences of fosmids selected during PCR-primer walking in the NFM2 fosmid library

Fosmid [a]	BLASTN (E-value)	Accession number	BLASTX (E-value)	Accession number
Upstream Fosmids [b]				
4G5-pCC2FOSF	*Acinetobacter sp*. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	Hypothetical protein A1S_3536 [*Acinetobacter baumannii* ATCC 17978] (1e-14)	ABS89961
4G5-pCC2FOSR	*Acinetobacter sp*. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	Hypothetical protein 4G5-orf33 [*Acinetobacter* sp. NFM2] (4e-71)	ACN73435
			Hypothetical protein 4G5-orf32 [*Acinetobacter* sp. NFM2] (5e-68)	ACN73434
5E8-pCC2FOSF	*Acinetobacter sp*. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	No significant matches	
5E8-pCC2FOSR	No significant matches		No significant matches	
1D8-pCC2FOSF	*Acinetobacter* sp. ADP1 complete genome (2e-10)	CR543861	Conserved hypothetical protein [*Acinetobacter johnsonii* SH046] (5e-38)	ZP_06062586
			Conserved hypothetical protein [*Acinetobacter johnsonii* SH046] (1e-22)	ZP_06062585
1D8-pCC2FOSR	*Acinetobacter* sp. ADP1 complete genome (1e-178)	CR543861	Sulfite reductase [*Acinetobacter johnsonii* SH046] (6e-100)	ZP_06062549
5G6-pCC2FOSF	No significant matches		No significant matches	
5G6-pCC2FOSR	*Acinetobacter* sp. ADP1 complete genome	CR543861	Putative transport protein (permease) [*Acinetobacter lwoffii* WJ10621]	ZP_09903660

	(6e-56)		(5e-42)	
2D10-pCC2FOSF 2D10-pCC2FOSR	No significant matches *Acinetobacter baumannii* ATCC 17978, complete genome (3e-148)	CP000521	No significant matches Transposase [*Acinetobacter baumannii* ACICU] (3e-60)	YP_001840868
1C9-pCC2FOSF 1C9-pCC2FOSR	No significant matches No significant matches		No significant matches No significant matches	
2A4-pCC2FOSF 2A4-pCC2FOSR	No significant matches No significant matches		No significant matches Hypothetical protein A1S_0635 [*Acinetobacter baumannii* ATCC] (2e-14)	YP_001083686
4A2-pCC2FOSF 4A2-pCC2FOSR	No Significant matches No Significant matches		No significant matches No significant matches	
3D3-pCC2FOSF 3D3-pCC2FOSR	No Significant matches No Significant matches		No significant matches No significant matches	
1G4-pCC2FOSF 1G4-pCC2FOSR	No Significant matches No Significant matches		No significant matches Conserved hypothetical protein [*Escherichia coli* MS 116-1] (4e-42)	ZP_07161693
4A1-pCC2FOSF	*Acinetobacter baumannii* MDR-ZJ06, complete genome (0.0)	CP001937	Hypothetical protein HMPREF0021_00602 [*Acinetobacter baumannii*] (5e-35) Transposase IS3/IS911 family protein [*Acinetobacter* sp. RUH2624] (1e-44) thiF family protein [*Brevibacillus laterosporus* GI-9] (3e-09)	ZP_08433031 ZP_05826607 CCF16463
4A1-pCC2FOSR	No Significant matches		Hypothetical protein A1S_3518 [*Acinetobacter baumannii* ATCC 17978] (9e-07)	ABS89943

Downstream Fosmids [c]

4B11-pCC2FOSF	*Acinetobacter* sp. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	IcmO [*Acinetobacter* sp. NFM2] (0.0)	ACN73408
4B11-pCC2FOSR	No Significant matches		Transglutaminase-like domain protein [uncultured *Leeuwenhoekiella* sp.] (2e-09)	CBL80578
2H7-pCC2FOSF	No significant matches		Unnamed protein product (1e-13)	YP_350937
			Conserved hypothetical protein [*Acinetobacter johnsonii* SH046] (2e-33)	ZP_06063651
2H7-pCC2FOS R	*Acinetobacter* sp. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	Hypothetical protein 4G5-orf28 [*Acinetobacter* sp. NFM2] (4e-137)	ACN73430
3E12-pCC2FOSF	No significant matches		Hypothetical protein (UBAL3_80150052) (3e-36)	EES53263
3E12-pCC2FOSR	Acinetobacter sp. NFM2 clone fosmid 4G5, complete sequence (0.0)	FJ711439	Hypothetical protein 4G5-orf28 [*Acinetobacter* sp. NFM2] (1e-74)	ACN73430
			Hypothetical protein Pecwa_0684 (6e-18)	YP_003258112
4F2-pCC2FOSF	No significant matches		No significant matches	
4F2-pCC2FOSR	No significant matches		No significant matches	

[a] Fosmids testing positive during various phases of the primer walking procedure. The fosmid identification number is given, followed by the vector primer used to generate the sequencing data. [b] Upstream fosmids identified by PCR primer walking of fosmid library. [c] Downstream fosmids identified by PCR primer walking of fosmid library.

6.4 - DISCUSSION

In previous chapters (4 and 5) we showed that an unusual class 1 integron is circulating among various species of *Acinetobacter* resident in the gut of wild prawns. The means by which the integron mobilizes between different *Acinetobacter* strains and species is not known. To investigate whether this mobility is mediated by plasmids or by other mobile elements, we investigated the genomic context of the class 1 integron. Fosmid libraries were constructed from *Acinetobacter* strain NFM2 (Gillings *et al.*, 2009) and used to extend the sequence surrounding the integron by primer walking. Sequence analysis of recovered fosmids revealed that most genes encoded hypothetical proteins previously described in diverse members of *Acinetobacter* (*A. lwoffii, A. johnsonii, A.sp*) or were unique, with no homology in the database (Table 6.1).

Several lines of evidence strongly suggest that the class 1 integron recovered in the present study is contained within a large genetic element which is probably located on the bacterial chromosome. No genes were recovered upstream or downstream of the integron which were indicative of it being a part of plasmid. In contrast, those protein homologies which could be identified were more typically chromosomally encoded. Nucleotide homologies were also stronger to *Acinetobacter* genome sequences generated from chromosomal DNA. Finally, we performed a total of 9 cycles of fosmid walking, and since the average insert length in the library is about 40 kb, the total span of sequence is likely to represent over 200 kb in total, with no sign of circularity (if this were the case, terminal PCRs for upstream fosmids should recover the distal downstream fosmids).

The integron/MITE element and all flanking regions were present in all integron positive *Acinetobacter* isolates from prawns, but missing in all negative strains. This suggests a

large contiguous DNA element is either present or absent in the strains. This same element was absent in all *Acinetobacter* isolates from clinical contexts, with one exception. A single isolate of *A.nosocomialis* was positive for all flanking PCRs, but negative for the integron and MITE elements. Since a sister isolate of *A. nosocomialis* was negative for all flanking PCRs, this strongly suggests that there is a large mobile DNA segment, perhaps of some 200 kb moving between *Acinetobacter* strains and species. In the case of isolates from prawns, this segment has captured the integron/MITE element.

Given evidence of mobility, such a large genetic element is probably a plasmid or genomic island. Several *Acinetobacter* strains harbor plasmid molecules of various sizes and these carry adaptive genes for pathogenicity, resistance to heavy-metals and antibiotics and biodegradation of hydrocarbons. Such plasmids have been correlated with the ability to adapt to fluctuating environmental conditions (Towner *et al.*, 1991; Bergogne-Berezin and Towner, 1996; Fondi *et al.*, 2010).

Likewise, Genomic islands can be found distributed on the chromosome of bacterial cell lineages in a strain specific manner (Juhas *et al.*, 2009). Large genomic islands containing diverse antibiotic resistance and virulence genes, metal resistance genes and transposition machinery are being increasingly identified from *Acinetobacter* isolated in clinical contexts (Fournier *et al.*, 2006; Krizova and Nemec, 2010; Post *et al.*, 2010; Krizova *et al.*, 2011). It has also been reported that many of the genes found in genomic islands are novel, with no detectable homologies, but that they might confer a selective advantage to the host cell (Hsiao *et al.*, 2005; Juhas *et al.*, 2009). This suggests a flexibility of the *Acinetobacter* genome, and an exceptional ability to gather foreign genetic material with potential fitness consequences.

However, DNA segment we have partially characterized here did not show homology to any previously described genomic islands. Thus, whole-genome sequencing of recovered *intI1* positive and negative strains will be needed to identify the extent of the genomic segment through which this unusual class 1 integron is mobilized between diverse lineages of *Acinetobacter.*

CHAPTER 7 - GENERAL CONCLUSION: THE HISTORY AND FUTURE OF THE CLASS 1 INTEGRON

Bacteria have a remarkable ability to adapt to hostile environmental conditions. The ability to survive under exposure to high levels of antibiotics is just the most recent of these pressures. The rapid proliferation of antibiotic resistance phenotypes across a wide variety of human commensals and pathogenic bacteria is commonly driven by lateral transfer of antibiotic resistance genes. This emergence and spread of antibiotic resistance among pathogens is a global problem, with significant repercussions for public health.

It is likely that the original source of resistance genes, and the DNA elements that mobilize them, lies in the natural environment. Recent studies have described the environmental "resistome" as a source of diverse resistance genes with the potential to become clinically important (D'Costa *et al.*, 2006; Martinez, 2008; Wright, 2010). Similarly, progress is being made on understanding the distribution and diversity of mobile DNA elements in natural ecosystems (Stokes and Gillings, 2011). Understanding the interactions between the resistome, bacterial diversity, and lateral gene transfer in natural and human-dominated environments is critical for managing the appearance of novel antimicrobial resistance determinants.

Mobilization of DNA elements, including resistance genes, predated the antibiotic era. Plasmids, transposons and integrons have a long evolutionary history, and have been documented as common features of bacterial strains collected before the use of antibiotics (Hughes and Datta, 1983; Jones and Stanley, 1992; Kholodii *et al.*, 2003; Mazel, 2006; Boucher *et al.*, 2007). However, the current human-dominated environment has high

concentrations of diverse selective agents that fix rare lateral transfer events, which consequently raises the abundance of both transferred genes and the cells containing them.

To better understand these processes and their impacts on human activities, we need to investigate the dynamics of lateral gene transfer at all levels, these being: the mobilized genes; the vectors that move these genes within and between cells; and the movement of bacterial cells between hosts and environments (Stokes and Gillings, 2011). In specific terms of antibiotic resistance, we need to understand the origins of resistance genes from the pool of such genes in the environment. We also need to understand the assembly of the diverse transposons, plasmids, and genomic islands that move resistance genes between lineages. Finally, we need to think about the ongoing consequences of lateral gene transfer and the dynamics of genes, vectors and cells that are released back into the environment from human-dominated ecosystems (Gillings and Stokes, 2012).

In this thesis, I have investigated the evolutionary history of the class 1 integrons. This class of integron is largely responsible for disseminating antibiotic resistance genes amongst Gram negative pathogens. I have also examined some of the second-order effects of the antibiotic era, particularly what might happen when cells containing class 1 integrons are released from human waste streams back into the general environment. The context and relationship of my work to the general research area is summarized below.

Integrons (Stokes and Hall, 1989) are gene capture and expression systems which have played a crucial role in the dissemination of antibiotic resistance genes. They have been described in a wide range of Gram negative, and more recently, in Gram positive bacteria (Martinez-Freijo *et al.*, 1998; Nandi *et al.*, 2004; Shi *et al.*, 2006; Xu *et al.*, 2011). Class 1

integrons are frequently identified from diverse lineages of environmental and pathogenic bacteria. Several studies have described the role of natural communities as a reservoir and original source of class 1 integrons (Stokes *et al*., 2006; Nandi *et al*., 2004; Nemergut *et al*., 2004). However, occurrence of class 1 integrons in general environments are subject to various degrees of human disturbance (Stokes *et al*., 2006; Nandi *et al*., 2004; Rosser & Young, 1999; Goldstein *et al*., 2001; Barlow *et al*., 2004; Nandi *et al*., 2004; Nemergut *et al*., 2004; Gaze *et al*., 2005). In general, it is assumed that some 2 to 3% of bacterial cells in non-clinical samples contain a class 1 integron (Hardwick *et al*., 2008). However, factors involved in the distribution or abundance of class 1 integrons outside clinical environments remain largely unknown. What is known is that they are capable of providing benefits to the host cell due to their ability to acquire gene cassettes that could provide a selective advantage for survival in a range of hostile environments (Stokes *et al*., 2001; Holmes *et al*., 2003; Michael *et al*., 2004; Gillings *et al*., 2005; Wright *et al*., 2008).

Class 1 integrons recovered from environmental samples have diverse integrase gene sequences, varied chromosomal locations and highly diverse gene cassettes, often of unknown function (Gillings *et al.*, 2008b). In contrast, class 1 integrons from pathogens and human commensals have a conserved integrase gene sequence, are commonly associated with a Tn*402* transposon backbone, and carry gene cassettes that primarily encode resistance functions (Partridge *et al.*, 2001; Stokes *et al.*, 2006; Gillings *et al.*, 2008a). The best explanation for these observations is that an environmental class 1 integron was captured by a Tn*402* transposon and that this hybrid element was the immediate ancestor of the class 1 integrons now so widely distributed amongst pathogenic bacteria. Surveys of environmental bacteria suggest that the original capture event involved a betaproteobacterial chromosomal integron, lacking resistance gene cassettes,

being inserted into a Tn*402*-like element (Stokes *et al.*, 2006; Gillings *et al.*, 2008a; Labbate *et al.*, 2008). Insertion of this transposon/integron hybrid into plasmid vectors via the *res*-hunting activity of Tn*402* (Kholodii *et al.*, 1995; Partridge *et al.*, 2002) then facilitated its movement among a broad range of bacteria.

Elements corresponding to most of the intermediate steps in the hypothesized evolution of clinical class 1 integrons have been discovered (Gillings *et al.*, 2008a). It has been established that class 1 integrons occur in range of environmental bacteria, and that their respective integrase genes exhibit considerable sequence diversity (Gillings *et al*., 2008b). These class 1 integrons often reside on the chromosomes of environmental betaproteobacteria, where they carry gene cassettes of unknown function (Fig. 7.1). Comparison between class 1 integrons in different species shows that they have a conserved sequences that mark the boundaries of the integron with the host chromosomal DNA. This in turn demonstrates that the class 1 integron is capable of mobility between locations and cells, at least over evolutionary time (Gillings *et al.*, 2008a).

It is hypothesized that a chromosomal class 1 integron of the type found in betaproteobacteria was mobilized from these boundary sequences, and captured by a Tn*402*-like transposon. A representative of the Tn*402* class of transposon, but without an embedded integron, has recently been recorded (Stokes *et al.*, 2006) (Fig. 7.1). This initial capture event therefore would have generated a Tn*402* transposon containing, in order: the terminal inverted repeat IRi; the class 1 integron left hand boundary sequence; the class 1 integron-integrase gene, *intI1*, with a sequence matching that found in antibiotic resistant isolates; a gene cassette array containing cassettes of unknown function, the class 1 integron right hand boundary sequence; an intact *tni* transposition module, and the terminal

inverted repeat IRt. It is this hybrid transposon/integron module that is hypothesized to be the immediate ancestor of all the mobile class 1 integrons found in human pathogens and commensals. However, prior to the work done in this thesis, no such structure had ever been recorded.

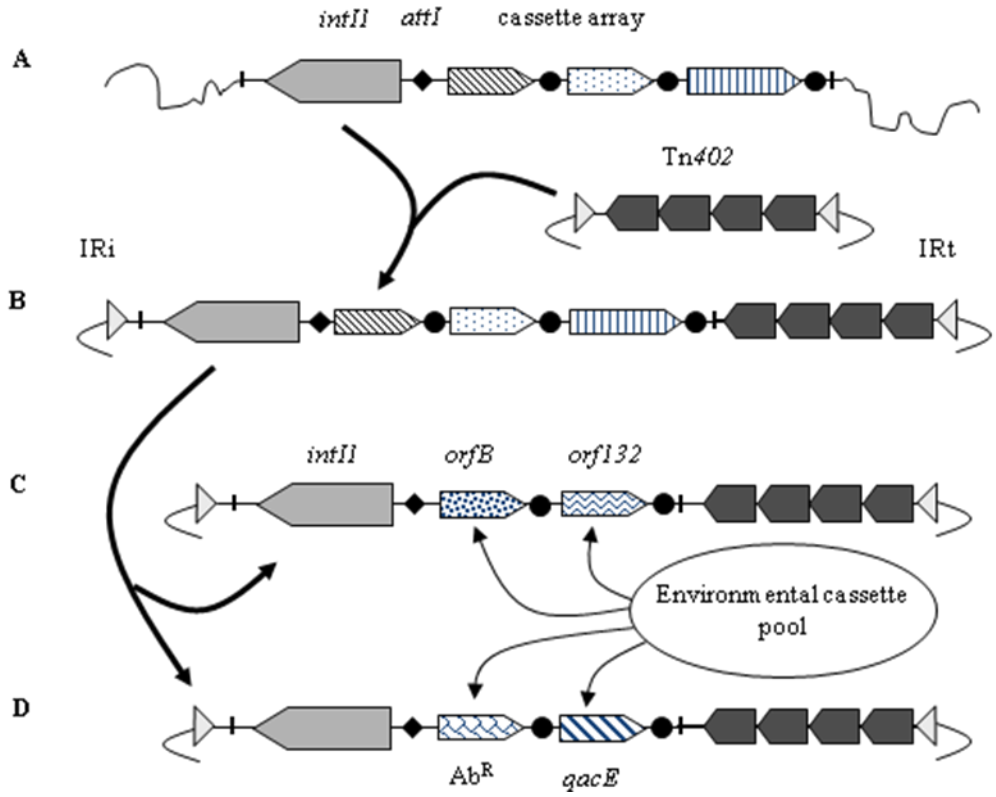


Fig. 7.1 Model for the origin of Tn*402* class1 integrons. (A) A class 1 integron resident in the chromosome of an environmental *Betaproteobacterium* is captured by a Tn*402* transposon to generate (B) a hybrid structure combining the ability of integrons to sample the environmental gene cassette pool with the enhanced mobility of the Tn*402* transposon. (C) The integron described in this thesis (Chapter 3), where the hybrid structure has targeted a resolvase gene resident in a *Pseudomonas* species, and the integron has acquired environmental genes cassettes, including one known to also be present in chromosomal class 3 integrons of *Delftia* (Xu *et al*. 2007). (D) One possible form of the ancestor of the class 1 integrons found in human pathogens and commensals. It contains an antibiotic resistance gene (Ab^R), a *qacE* gene cassette conferring resistance to quaternary ammonium compounds and a complete *tni* transposition module (Sajjad *et al.*, 2011).

In this study, we present evidence that a class 1 integron with this hypothetical ancestral structure (Stokes *et al.*, 2006; Gillings *et al.*, 2008a) is still circulating in the general environment. The class 1 integron/Tn*402* hybrid was recovered in a *Pseudomonas* species isolated from the gut flora of a wild-caught Eastern king prawn (*Panaeus plebejus*) (Sajjad *et al.*, 2011). Its structure represents an example of a key intermediate in the evolution of the clinical class 1 integron, which was previously predicted but never observed (Gillings *et al.*, 2008a). Also as predicted, the gene cassettes in this integron did not encode antibiotic resistance determinants, but encoded hypothetical proteins. The presence of a cassette that was identical to a cassette previously recovered from a chromosomal class 3 integron in *Delftia* (Xu *et al.*, 2007) demonstrates that cassettes are readily exchanged between different classes of integrons in natural environments. Consequently, this ancestral class 1 integron has the ability to freely acquire gene cassettes from the enormous and diverse pool of gene cassettes known to be present in environmental samples (Fig. 7.1) (Michael *et al.*, 2004; Koenig *et al.*, 2008). This presumably included gene cassettes that confer phenotypes that would be useful in surviving antimicrobial exposure, including resistance to disinfectants and antibiotics.

The first gene cassette likely to have conferred a selective advantage in a clinical setting is thought to have been the *qacE* cassette (Gillings *et al.*, 2008c). There are a number of reasons for suspecting this. Firstly, about half of all class 1 integrons from environmental samples contain *qac* cassettes as part of their arrays (Gillings *et al.*, 2008c), and so there is a 50% chance that the initial integron capture event included a *qac* gene. Secondly, a deleted version of *qacE*, fused to the *sul1* gene, is a consistent feature of most class 1 integrons recovered from clinical settings. Indeed, this fusion forms part of the 3' conserved segment (3'-CS) of clinical class 1 integrons, showing that *qac* and *sul* are

ancestral parts of the clinical class 1 integron (Stokes and Hall, 1989; Hall *et al.*, 1994). Thirdly, the likely timing of the appearance of clinical class 1 integrons coincides with the first use of quaternary ammonium compounds in clinical settings, thus providing a selective agent capable of fixing the Tn*402*/integron hybrid in human commensals or pathogens (Gillings *et al.*, 2008c).

The evolutionary history of the clinical class 1 integron, and its penetration into a wide variety of commensals and pathogens can now be reconstructed with some confidence (Fig. 7.2). Initially, a chromosomal integron was captured by a Tn*402* transposon. Its gene cassette array probably contained a *qacE* cassette in the distal position, and selection for resistance to quaternary ammonium compounds fixed this initial event and any subsequent lateral transfers into various cell lineages. Insertion of the *sul1* gene deleted the terminal part of the *qacE* cassette, and provided a further resistance phenotype for selection to act upon. Deletions of various kinds rendered the *tni* transposition module inactive for transposition, but the *res* hunting ability of Tn*402* had already spread the transposon/integron to a number of plasmid vectors, thus ensuring its dissemination between cells and species. The class 1 integron-integrase was active, and able to sample the environmental gene cassette pool, thus fixing various cassettes encoding antibiotic resistance in diverse sub-lineages, and in different species of human commensal and pathogenic flora. Continued accumulation of resistance cassettes and extensive lateral transfer has led to the current situation, where up to 70% of Gram negative pathogens carry class 1 integrons (Martinez-Freijo *et al.*, 1998; van Essen-Zandbergen *et al.*, 2007), as do many commensal flora of both humans and their domesticates (Goldstein *et al.*, 2001; Ebner *et al.*, 2004; Labbate *et al.*, 2008).

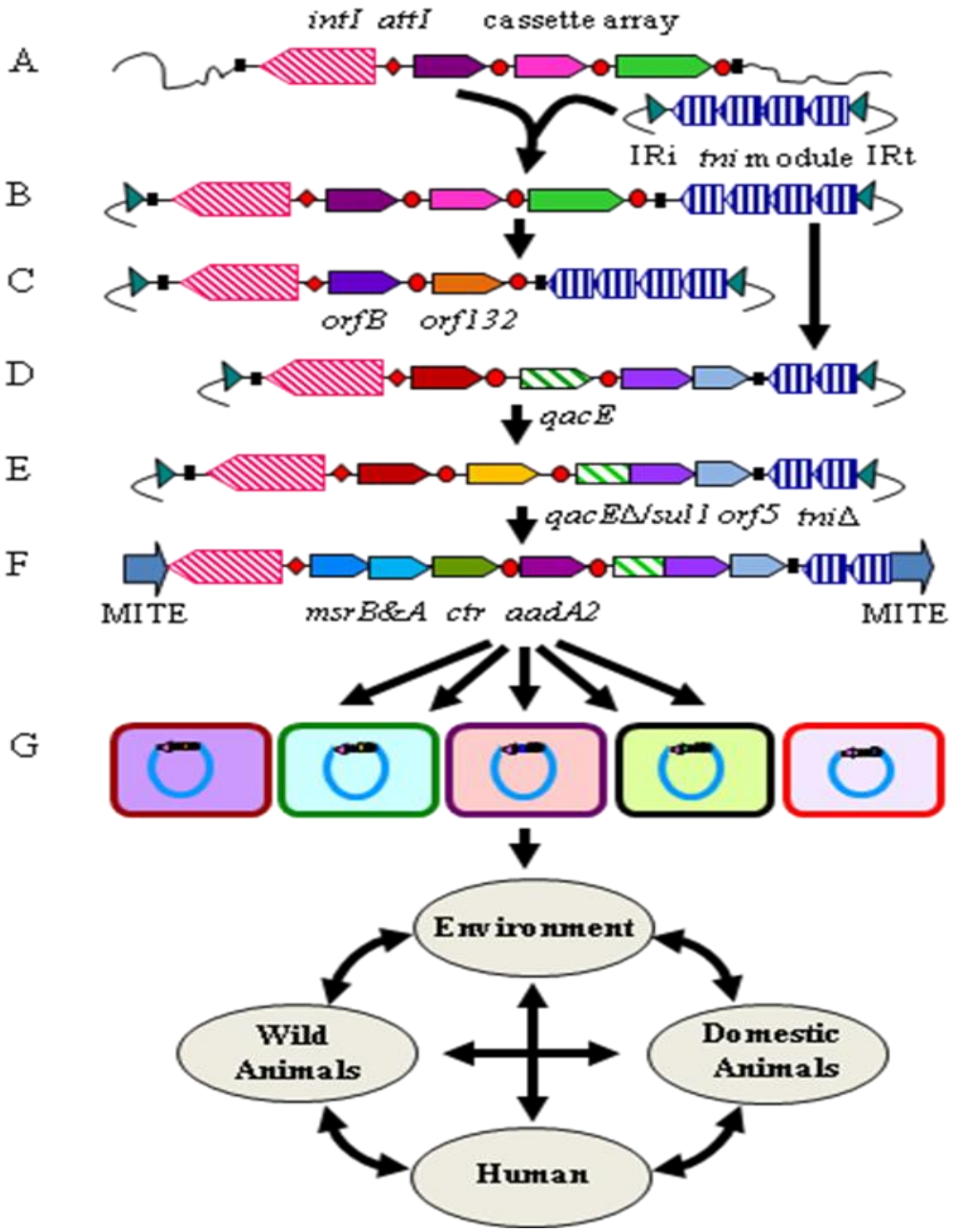


Fig. 7.2 Model for the evolutionary history of the clinical class 1 integron and its penetration into diverse commensals and pathogenic bacteria. (A) A chromosomal class 1 integron of *Betaproteobacterium* is captured by Tn*402* transposon creating (B) the integron/transposon hybrid structure with enhanced ability for lateral transfer coupled with the ability to recruit gene cassettes from the environmental cassette pool. (C) The class 1

integron representing a descendant of original capture event, containing environmental gene cassettes and a complete transposition (*tni*) module, as recovered during this study (Chapter 3). (D) The potential direct ancestor of clinical class 1 integrons, containing a *qacE* gene cassette encoding resistance to quaternary ammonium compounds and complete transposition (*tni)* machinery. (E) Insertion of the *sul1* and orf5 genes, resulting in deletion of terminal part of *qacE* gene cassette, and subsequent partial deletion of the *tni* module, thus creating the 3'-CS. (F) The class 1 integron/MITE structure recovered during this study, representing the ongoing evolution of the clinical class 1 integron. This integron has acquired a novel means of mobility via the flanking MITEs and acquired a novel gene cassette encoding a potential pathogenicity factor (*msr*). (G) Spread of the novel class 1 integron into diverse strains and species of *Acinetobacter*. We anticipate that this integron and its derivatives will also spread to further environmental organisms, commensals and pathogens, and that the interchange of bacterial cells, vectors and mobile genes will continue to generate new complexity in lateral transfer elements of relevance to human welfare.

The high frequency of clinical class 1 integrons in human flora means that large numbers of these elements are being shed from human-dominated ecosystems. Descendants of the original capture event are being constantly disseminated back into the environment via human waste streams. Human activity releases a complex mixture of DNA elements, commensals and pathogens containing resistance determinants into the environment, along with a range of selective agents such as antimicrobials, detergents and heavy metals. This creates hotspots for interactions between plasmids, integrons, transposons and genomic islands, and the various cells and species that might benefit from acquiring resistance determinants (Moura *et al.*, 2010; Stokes and Gillings, 2011; Gillings and Stokes, 2012). Thus it is likely that a diverse range of environmental organisms will acquire class 1 integrons and will consequently have access to the pool of gene cassettes known to exist in natural environments. Since we do not have a detailed knowledge of the selection pressures present in such environments, it is uncertain what the consequences might be of the wholesale release of resistance genes and their vectors, coupled with a range of selective agents. Consequently, we aimed to investigate if class 1 integrons with clear clinical/human commensal ancestry had established themselves in environmental niches, and if so whether they had fixed additional lateral transfer events.

To conduct this study, we chose to investigate wild prawns, on the basis that these animals are filter feeders with close ties to near-shore environments, and are likely to come in contact with bacteria and DNA vectors emanating from human waste streams. We isolated bacteria from the digestive systems of wild-caught prawns and screened these for the presence of integrons. More than 75% of prawns examined were positive for *intI1* in mixed cultures of their gut contents. We isolated pure *intI1* positive cultures for further characterization of the integrons and the cells that contained them.

The first investigations focussed on a class 1 integron from *Acinetobacter johnsonii* (Gillings *et al.*, 2009) isolated from the digestive flora of a wild Eastern king prawn. This class 1 integron possessed features typical of a Tn*402*-like class 1 integron. It carried a typical 3' conserved segment, consisting of a *qacE/sul1* fusion and truncated *tni* module, and its *intI1* sequence was identical to that of other class 1 integrons isolated from clinical contexts. Additionally it carried a gene cassette encoding the known antibiotic resistance gene *aadA2*, which confers resistance to streptomycin and spectinomycin. These features clearly identify the integron as originating from the human environment.

However, the integron exhibited some unusual features not previously found in class 1 integrons. Firstly, the terminal repeats IRi and IRt that define the boundaries of the Tn*402* transposon had been replaced by miniature inverted repeat transposable elements (MITEs). Secondly, the integron had acquired a novel gene cassette, not previously recorded in any integron. This cassette contained *msr* genes, responsible for repairing oxidative damage to methionine residues (Grimaud *et al.*, 2001; Gillings *et al.*, 2009).

We concluded that a typical class 1 integron in a clinical or commensal bacterium had been released via human waste streams into the environment, where it had been taken up during filter feeding by an eastern king prawn. Sometime during this process, the integron had transferred into the *Acinetobacter johnsonii* strain now resident in the prawn gut, and various modifications had taken place, including the incorporation of MITEs and the novel gene cassette. The proximal position and novelty of the *msr* gene cassette suggested that it conferred some selective advantage within the invertebrate digestive system. It is not possible to determine where or when each of these events occurred, since we can only observe the end result.

Miniature inverted repeat transposable elements (MITEs) belong to a family of non-autonomous mobile elements (Delihas, 2008). This family is diverse with respect to both sequence and properties, with no universal defining features (Delihas, 2008). Pairs of MITEs that flank a region of DNA can move such a region through simultaneous transposition, or can move DNA by recombination with other homologous MITEs. The association of class 1 integrons with yet another family of mobile elements adds to the potential paths of lateral gene transfer and recombination that can spread integrons into diverse species and environments. A study in Portugal reported MITE-like structures in association with a class 1 integron from the opportunistic pathogen *Acinetobacter baumannii* isolated from a clinical context (Domingues *et al.*, 2011). Interestingly, this MITE element showed 100% nucleotide identity to the MITE element recovered during our study, although the gene cassette arrays of the respective integrons were entirely different. More recently, the same group has reported the identification and characterization of MITE like structure from seven clinical *Acinetobacter* spp. isolated in Portugal and Brazil (Domingues *et al*., 2013). This observation suggests that MITEs are becoming more commonly associated with integrons, and consequently are adding to the potential for their rapid dissemination between species. It is particularly concerning that integron/MITE combinations should be found in various strains of *Acinetobacter* isolated from both clinical and non-clinical environments, given the emerging importance of this genus as an opportunistic pathogen (Gillings *et al.*, 2009; Towner, 2009; Domingues *et al.*, 2011; Visca *et al.*, 2011). Recently, a class 1 integron also flanked by MITEs has also been identified in a clinical *Enterobacter cloacae* isolate, although these MITEs were not related in sequence to those found here (Poirel *et al.*, 2009).

To further examine the potential transmission of MITEs and integrons in the environment, we screened additional prawns for *intI1* positive isolates. Identical class 1 integrons were identified in various species of *Acinetobacter* recovered from prawns, including additional isolates of *Acinetobacter johnsonii,* from *A. lwoffii* and from an unnamed *Acinetobacter* species. Using DNA fingerprinting, we showed that the isolates within any one species were not clonal, and consequently, the MITE/integron element had been laterally transferred between a number of distinct strains within species, and to multiple strains within at least another two species of *Acinetobacter*. Further, the original prawn samples were collected from a geographical range representing over 2,500 km of the south east coast of Australia. Taken in total, these data establish that this unusual class 1 integron has been actively exchanged between members of the genus *Acinetobacter* in this aquatic ecosystem.

These results help us to understand the consequences of the wholesale release of resistance genes and their vectors from human dominated ecosystems. Here we have a clear example of a class 1 integron, emanating from a clinical or commensal source, making its way back into the general environment. In this new environment it has acquired a novel form of mobility in terms of the MITE elements, and has acquired a previously undescribed gene cassette with a potential function in survival within a metazoan digestive system. Furthermore, this genetic element is being readily transferred between strains and species of *Acinetobacter*.

Members of the genus *Acinetobacter* are emerging opportunistic pathogens responsible for serious nosocomial infections (Wisplinghoff *et al.*, 2000; Mammeri *et al.*, 2003). The genus is generally considered to be ubiquitous, since it is frequently identified from soil,

water, food and clinical environments (Guenthner *et al.*, 1987; Gennari and Lombardi, 1993; Seifert *et al.*, 1997; Guardabassi *et al.*, 1999). Various *Acinetobacter* species, including *A. johnsonii* and *A. lwoffii*, are frequently isolated from human skin. Consequently, integron containing strains from prawn have a ready means of transferring the integron and associated DNA into human commensals and to known opportunistic pathogens in *Acinetobacter*. Recent studies have described the movement of class 1 integrons between diverse environmental and clinical isolates of *Acinetobacter* (e.g. *A. johnsonii, A. pittii* and *A. baumannii*) (Seifert *et al.*, 1997; Yamamoto *et al.*, 2011). From these observations it appears that these species could play an important role in potential transfer of genetic information between clinical and natural environments.

PCR mapping of the flanking regions surrounding the class 1 integron from *Acinetobacter* revealed that the MITE-integron-MITE structure was probably embedded in a much larger DNA segment that was being mobilized between diverse environmental *Acinetobacter* strains and species. Large genomic islands coupled with diverse transposons, integrons and metal resistance genes have been previously reported from *Acinetobacter* strains isolated from clinical contexts (Fournier *et al.*, 2006; Krizova and Nemec, 2010; Post *et al.*, 2010; Krizova *et al.*, 2011). However, the DNA region we have partially characterized here did not show homology to any previously described genomic islands.

The DNA region immediately surrounding the MITE-integron element was also detected in an opportunistic pathogen, *A. nosocomialis* (Nemec *et al.*, 2011) but in this case the integron/MITE structure was missing. This suggests that the genomic segment which was subject to transfer between *Acinetobacter* species resident in the prawn gut was also

present in at least one clinical isolate. This makes it even more likely that the MITE-integron will make its way into nosocomial *Acinetobacter* species in the near future.

This part of the current work followed the fate of a typical Tn*402*-like class 1 integron, containing a 3'-CS and a gene cassette encoding antibiotic resistance, after it was released into the general environment. In the natural environment, the integron underwent further evolution and modification, having acquired a gene cassette (*msr*) that encoded a potential pathogenicity determinant, and replacing the terminal repeats IRi and IRt by MITE elements. The MITE elements provide a means of transposition not available to the original integron, which was embedded in the defective Tn*402* backbone. It appears that the MITE-integron hybrid was subsequently captured by a larger mobile genetic element, which was then extensively disseminated between multiple strains of *Acinetobacter* resident in the digestive flora of prawns. It seems likely that this larger element is a previously undescribed genomic island.

In conclusion, our research demonstrates the interconnections between human commensals/pathogens, environmental microorganisms, and their hosts. DNA elements of various kinds can clearly be shared between all these cells and environments across very short evolutionary time frames. There are clear implications for human health in the specific instances we have documented. The unusual class 1 integrons we describe were isolated from genera known to be emerging pathogens (*Acinetobacter* sp. and *Pseudomonas* sp.). These bacteria were resident in the gut flora of prawns, which are an important food source, widely caught and farmed for human consumption. They are often prepared with only light cooking, providing a clear pathway for bacteria and their mobile elements to interact with the human commensal flora, and to transfer novel DNA elements.

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