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FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

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Unravelling novel mechanisms of horizontal transfer of class 1 integrons in bacteria

Implications for the dissemination of antibiotic resistance

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Abstract

Mobile genetic elements (MGEs), such as transposons and integrons, frequently carry antimicrobial resistance determinants and can be found widely disseminated among pathogenic and non-pathogenic bacteria. Their distribution pattern suggests dissemination through horizontal gene transfer. The role of natural transformation in horizontal transfer of genetic elements other than those that are self-replicative (plasmids) has remained largely unexplored. In this work, we aimed to determine the potential for class 1 integrons and gene cassettes to move horizontally between bacterial species via natural transformation, and reveal any genetic linkage between the acquired integrons and MGEs.

We have screened the presence of class 1 integrons among clinical and animal bacterial collections, as well as determined the genetic context of some of the integrons. We investigated to what extent natural transformation acting on free DNA substrates can facilitate the transfer of mobile elements, including transposons, integrons and/or gene cassettes between bacterial species. Naturally transformable cells of *Acinetobacter baylyi* were exposed to DNA from class 1 integron-carrying strains of the genera *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Pseudomonas*, and *Salmonella* to determine the nature and frequency of transfer. Additionally, the host fitness burden imposed by the transferred class 1 integron-containing DNA was determined.

A high prevalence of class 1 integrons was detected among the clinical (40.7 %) and animal (14.5 %) bacterial isolates screened. All the class 1 integrons for which the flanking regions were analysed were associated with transposable elements, including transposons, insertion sequence (IS)-composite transposons and miniature inverted repeat transposable elements (MITEs). Exposure of *A. baylyi* cells to the different DNA sources over a 24 hrs exposure period resulted in acquisition of antibiotic resistance traits as well as entire class 1 integrons and transposons. DNA incorporation was largely independent of the genetic relatedness between species. DNA sequence analyses revealed that

different mechanisms facilitated stable integration in the recipient genome depending on the nature of the donor DNA, including homologous recombination and transposition of transposons and composite-transposons. Both donor strains and transformed isolates were extensively characterized by antimicrobial susceptibility testing, class 1 integron- and gene cassette-specific PCRs, DNA sequencing, pulsed field gel electrophoreses (PFGE), Southern blot hybridizations, and by re-transformation assays. Additionally, three transformant isolates were genome-sequenced. Our data demonstrate that natural transformation facilitates interspecies transfer of genetic elements, suggesting that the transient presence of DNA in the cytoplasm may be sufficient for genomic integration to occur. Our study provides a plausible explanation for why sequence-conserved transposons, IS elements and integrons can be found disseminated among bacterial species. In addition to class 1 integrons acquisition, natural transformation of *A. baylyi* with different species-foreign DNA resulted in the obtainment of transformants with altered antimicrobial susceptibility, without an obvious resistance gene acquisition. These observations suggest that natural transformation might result in the uptake of a wide range of DNA fragments, which contribute to the generation of antimicrobial resistance in bacteria. Natural transformation of class 1 integron harbouring populations of competent bacteria revealed that interspecies exchange of gene cassettes can be highly efficient, and independent on genetic relatedness between donor and recipient. Furthermore, class 1 integrons and flanking regions acquired by natural transformation did not impose a significant fitness cost to the transformant cell, which mean they might be maintained even in the absence of antibiotic pressure.

In conclusion, natural transformation provides a much broader capacity for horizontal acquisitions of genetic elements and resistance traits from divergent species than previously assumed. In particularly, natural transformation contributes to the dissemination of class 1 integrons, with consequent spread and maintenance of antimicrobial resistance genes among the bacterial communities.

Resumo

Elementos genéticos móveis (EGMs), tais como transposões e integrões, possuem frequentemente determinantes de resistência antimicrobiana, estando amplamente disseminados entre bactérias patogénicas e não-patogénicas. O padrão de distribuição de tais elementos sugere a sua disseminação através da transferência horizontal. O papel da transformação natural na transferência horizontal de elementos genéticos que não são auto-replicativos (plasmídeos) permanece amplamente inexplorado. O objectivo deste trabalho foi determinar o potencial dos integrões de classe 1 e dos genes-cassete para se moverem horizontalmente entre espécies bacterianas através da transformação natural, e avaliar possíveis relações genéticas entre os integrões adquiridos e os EGMs. A presença de integrões de classe 1 foi pesquisada em isolados bacterianos clínicos e de animais, assim como foi determinado o ambiente genético de alguns desses integrões. Investigámos até que ponto a transformação natural, actuando sobre substratos de ADN transformante, pode facilitar a transferência de elementos móveis, incluindo transposões, integrões e/ou genes-cassete, entre espécies bacterianas. A espécie *Acinetobacter baylyi*, naturalmente transformável, foi exposta a ADN de estirpes contendo integrões de classe 1 pertencentes aos géneros *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Pseudomonas* e *Salmonella*, de modo a determinar a natureza e a frequência da transferência. Além disso, foi determinado o custo biológico imposto aos transformantes pelo ADN adquirido, contendo um integrão de classe 1.

Uma elevada prevalência de integrões de classe 1 foi detectada entre os isolados bacterianos de origem clínica (40.7 %) e animal (14.5 %). Todos os integrões de classe 1 para os quais se determinou o contexto genético no qual estão inseridos revelaram uma associação com elementos transponíveis, incluindo transposões, transposões compostos e MITEs. A exposição de células de *A. baylyi* às diferentes fontes de ADN por um período de exposição de 24 hrs resultou tanto na aquisição de características de resistência a antibióticos como de integrões de classe 1 e transposões. A incorporação de

ADN nas células receptoras foi largamente independente da relação genética entre as espécies bacterianas envolvidas. A análise do ADN sequenciado revelou que diferentes mecanismos contribuíram para a sua integração estável no genoma da bactéria receptora, dependendo do ADN da dadora, e incluíam recombinação homóloga e transposição de transposões e transposões compostos. Tanto as estirpes dadoras como os transformantes foram extensamente caracterizados através da determinação da susceptibilidade antimicrobiana, PCRs específicos para integrões de classe 1 e genes-cassete, sequenciação de ADN, electroforese de gel em campo pulsado, hibridizações Southern blot e ensaios de re-transformação. Adicionalmente, o genoma de três transformantes foi completamente sequenciado. Os nossos resultados demonstram que a transformação natural facilita a transferência interespecies de elementos genéticos, sugerindo que a presença transitória de ADN no citoplasma poderá ser suficiente para haver integração no genoma. O nosso estudo proporciona uma explicação plausível para a existência de transposões, sequências de inserção e integrões com sequência conservada amplamente disseminados entre diferentes espécies bacterianas. Para além da aquisição de integrões de classe 1, a transformação natural de *A. baylyi* com vários ADNs de outras espécies resultou na obtenção de transformantes com susceptibilidade antimicrobiana alterada, sem que houvesse aquisição de genes de resistência identificáveis. Estas observações sugerem que a transformação natural poderá resultar na captação de uma grande variedade de fragmentos de ADN, contribuindo para a geração de resistência antimicrobiana nas bactérias. A transformação natural de populações bacterianas competentes contendo integrões de classe 1 revelou que a troca interespecies de genes-cassete pode ser altamente eficiente e independente da relação genética entre as bactérias receptora e dadora. Para além disso, os integrões de classe 1 e respectivas zonas adjacentes adquiridas através de transformação natural não impuseram aos transformantes um custo biológico significativo, o que significa que os integrões poderão ser mantidos mesmo na ausência de uma pressão antimicrobiana selectiva.

Em conclusão, a transformação natural fornece uma capacidade de aquisição horizontal de elementos genéticos e de características de resistência entre espécies bacterianas mais ampla do que o inicialmente assumido. Particularmente, a transformação natural contribui para a disseminação de integrões de classe 1, com consequente propagação e manutenção de genes de resistência aos antibióticos em comunidades bacterianas.

Abbreviations

3'-CS	3'-conserved segment
5'-CS	5'-conserved segment
AFLP	Amplified fragment length polymorphism
AM	Ampicillin
ARDRA	Amplified ribosomal DNA restriction analysis
AK	Amikacin
bp	Basepairs
BSA	Bovine serum albumine
CAZ	Ceftazidime
cDNA	Complementary DNA
CDS	Coding DNA sequence
CFU(s)	Colony-forming unit(s)
CI(s)	Chromosomal integron(s)
Cl	Chloramphenicol
CLSI	Clinical Laboratory Standards Institute
CN	Gentamicin
CTn(s)	Conjugative transposon(s)
CTX	Cefotaxime
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dsDNA	Double-stranded DNA
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin Methylene Blue
GI(s)	Genomic island(s)
HFIR	Homology-facilitated illegitimate recombination
HGT	Horizontal gene transfer

hr(s)	Hour(s)
ICE(s)	Integrative and conjugative element(s)
IMP	Imipenem
IMU	Integron mobilization unit
IR(s)	Inverted repeat(s)
IS(s)	Insertion sequence(s)
ISCR(s)	Insertion sequence common region(s)
K	Kanamycin
kb(s)	Kilobase(s)
LB	Luria-Bertani
M	Malthusian
Mb	Megabasepairs
MEM	Meropenem
MEPS	Minimum efficient processing segment
MGE(s)	Mobile genetic element(s)
MI(s)	Mobile integron(s)
MIC(s)	Minimal inhibitory concentration(s)
min	Minute(s)
MITE(s)	Miniature inverted repeat transposable element(s)
MTn(s)	Mobilisable transposon(s)
NET	Netilmicin
OD	Optical density
ON	Overnight
PBP(s)	Penicillin-binding protein(s)
PCR	Polymerase chain reaction
PDM	Paper disc method
PFGE	Pulse-field gel-electrophoresis
R	Rifampicin
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rpm	Rotations per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction

S3	Compound sulphonamides
SC	Spectinomycin
SD	Sulfadiazine
SDS	Sodium dodecyl sulphate
sec	Second(s)
SI(s)	Superintegron(s)
SM	Streptomycin
SOB	Super optimal broth
SOC	Super optimal catabolite repression
SOS	Damage-inducible cellular response
SSC	Saline-sodium citrate
ssDNA	Single-stranded DNA
Suc	Sucrose
SX	Sulphamethoxazole
SXT	Sulphamethoxazole/trimethoprim
T _a	Annealing temperature
TAE	Tris-acetate-EDTA buffer
TB	Transformation buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
T _m	Melting temperature
Tn(s)	Transposon(s)
TOB	Tobramycin
TSD(s)	Target site duplication(s)
UV	Ultraviolet
VIS	Visible
vol	Volume
W	Trimethoprim

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Introduction

Horizontal gene transfer (HGT) contributes to the genetic diversity and evolutionary trajectories of bacterial populations. In particular, HGT of mobile genetic elements (MGEs) is a major contributor to the emergence, recombination and dissemination of multidrug resistance among bacterial pathogens (Nakamura et al., 2004; Thomas and Nielsen, 2005). MGEs are often shared between bacterial species and separately evolving lineages due to their capacity to physically move within genomes, and also between host genomes and cytoplasms. A variety of MGEs have been described so far (Stokes and Gillings, 2011).

Integrans are genetic elements that contain a site-specific recombination system able to capture, express and exchange specific DNA elements, called gene cassettes (Hall and Collis, 1995). The complete integron is not considered to be a mobile element as such as it lacks functions for self-mobility. In contrast, the gene cassettes present in integrans are considered mobile within genomes, although the frequencies and modes of exchange of cassettes are rarely observed experimentally (Guerin et al., 2009; Baharoglu et al., 2010). Nevertheless, sequence similar integrans appear to be widespread among bacterial species and genetic backgrounds, suggesting that they are frequently exposed to mechanisms that allow them to disseminate horizontally through bacterial populations (Stokes and Hall, 1989).

I. Horizontal Gene Transfer

In contrast to vertical gene transfer from the parental generation to offspring, lateral or horizontal gene transfer (HGT) results in the transfer of genetic material between bacteria of the same generation, genetically related or not. Gene exchange by HGT acts in combination with natural selection of the newly created genetic diversity, and subsequent mutations, gene loss, and other chromosomal alterations, resulting in bacterial adaptive evolution. HGT allows bacteria to evolve in a way that would not be possible only by vertical

inheritance. Thus HGT is a major contributor to bacterial biodiversity, allowing prokaryote to rapidly alter their phenotypic properties by acquiring new genes and functions (Baptiste et al., 2005; Skippington and Ragan, 2011). Compositional analyses of bacterial genomes have revealed that a significant proportion consists of genes that have, at some time point, been acquired horizontally (Nakamura et al., 2004; Ochman et al., 2005; Thomas and Nielsen, 2005).

Several factors can limit the occurrence of HGT between bacteria (Jain et al., 2003). The barriers that contribute to maintain the genetic isolation of bacterial species include the genetic incompatibility, physical proximity and characteristics of the microhabitats, the host ranges of genetic exchange vectors, activity of host restriction-modification systems, as well as constraints to homologous recombination caused by DNA sequence divergence and functional incompatibilities (Matic et al., 1996; Thomas and Nielsen, 2005). Importantly, an evolutionary successful HGT does not rely only on the introduction and integration of DNA into a recipient bacterium, but also on long-term stability and maintenance of the transferred sequences over bacterial generations (Ochman et al., 2000).

HGT between bacteria is known to occur by at least three different processes: conjugation, transduction and natural transformation (Figure 1). These intercellular gene transfer mechanisms are based on transfer of DNA directly from a donor cell by conjugation, indirect transfer by donor cell release of bacteriophages, or indirectly by release of fragmented donor DNA. Thus, some modes of HGT do not require physical connection between the two cells involved. The resulting cell is called a transformed cell. Both in transduction and in natural transformation, the transferred DNA, if chromosomal, must be able to recombine with the recipient host. Such requirement often limits the horizontal transfer of chromosomal DNA fragments to members of the same bacterial species or closely related bacteria (Frost et al., 2005).

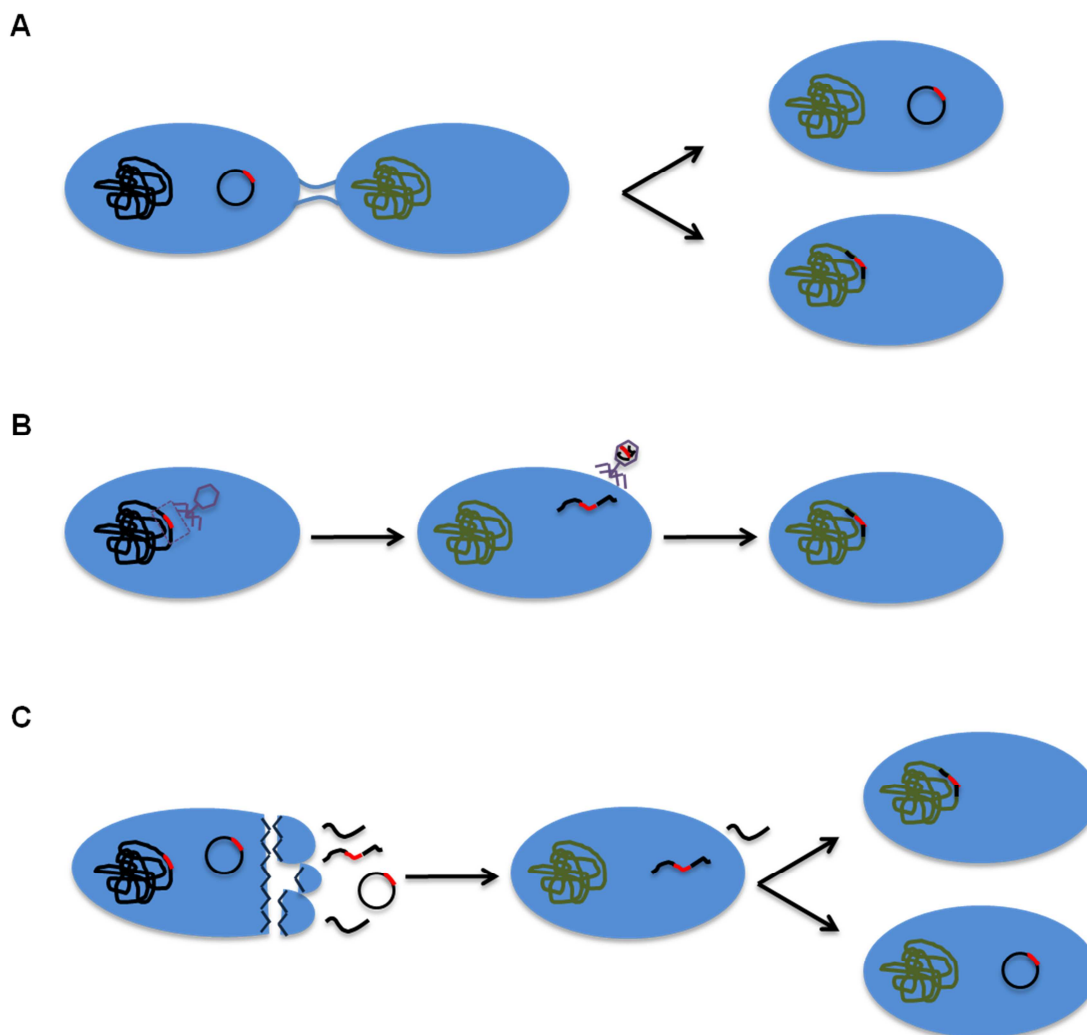


Figure 1. Schematic representation of the three classic HGT mechanisms. A – Conjugation; B – Transduction; C – Natural transformation. The red segment represents the horizontally transferred trait.

1. Conjugation

Conjugation is characterized by the transfer of genetic material between two bacteria in direct physical contact. It is usually a replicative process resulting in copy(ies) of the transferred material in both participating cells (Figure 1A). The donor cell can transfer conjugative plasmids, and integrative and conjugative elements (ICEs). Moreover, mobilisable plasmids, as well as chromosomal DNA, can hitchhike with co-resident conjugative plasmids, and therefore

transfer by conjugation (Ochman et al., 2000; Skippington and Ragan, 2011). Conjugation can transfer DNA of hundreds to thousands of kilobases (kbs) (Matic et al., 1996), which means that many traits can be transferred simultaneously.

Successful conjugation requires compatibility between the donor and recipient cells. The conjugation frequency depends primarily on the donor cell, although factors in the recipient cytoplasm can limit the entry or establishment of incoming DNA (Thomas and Nielsen, 2005; Skippington and Ragan, 2011). For instance, plasmids that belong to the same incompatibility (Inc) group use a common mechanism for replication or transfer and can therefore not co-exist in the same cell (Skippington and Ragan, 2011). The factors that determine the exact host range of plasmids are not clear, but one factor can be related with the presence of surface receptors on the recipient cell (Bennett, 2008).

2. Transduction

Transduction is the transfer of bacterial genes by viruses, called bacteriophages or phages (Figure 1B). When such phages are integrated in the host bacterial chromosomal DNA, they are denominated prophages. During transduction, some host cell DNA can be accidentally incorporated into the phage capsid together with the virus DNA, and then become injected into another bacterium. Such DNA can recombine with the host chromosome provided sufficient DNA similarity. In generalized transduction, any DNA fragment can be transferred, while in specialized transduction, only specific genes, located near the site of the prophage insertion site, can be transferred (Ochman et al., 2000). The amount of DNA that can be transferred during transduction depends on the size of the phage capsid, but can range from tens to hundreds of kbs (Matic et al., 1996). The ability to transduce host DNA seems to be limited to relatively large double-stranded DNA phages (Frost et al., 2005).

Both chromosomal and plasmids can be transferred by transduction (Davison, 1999). Not all phages are able to transduce and not all bacteria are

transducible. The host range of phages is determined by the specificity of the interaction between the bacteriophage and bacterial receptor sites (Mazodier and Davies, 1991; Ochman et al., 2000).

3. Natural transformation

Natural transformation is characterized by the incorporation of naked DNA from the environment into a recipient cell (Figure 1C); this includes the uptake, integration and functional expression of the extracellular DNA (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005). Both chromosomal and plasmid DNA can be taken up by transformation. However, the transformation of plasmids is less efficient, as it requires complex steps in the cytoplasm to reassemble a circular duplex molecule from the DNA fragments taken up over the bacterial membrane(s) (Thomas and Nielsen, 2005; Skippington and Ragan, 2011). Natural transformation has the potential to promote exchange of DNA among taxonomically diverse bacteria (Ochman et al., 2000), though the need of homologous recombination with the recipient genome might be a limiting factor (Frost et al., 2005). A successful integration of the incoming chromosomal DNA into the host genome is expected only if it is less than 30 % divergent from the recipient genome (Nielsen et al., 2009). Transformation with plasmid DNA excludes the need of a close genetic relationship between the donor and recipient cells (Lorenz and Wackernagel, 1994).

DNA can be released into the environment after bacterial lysis or by excretion by living cells (Thomas and Nielsen, 2005). Such DNA is easily fragmented, and only a small proportion is likely to be exposed to recipient bacteria (Matic et al., 1996; Nielsen et al., 2007). For most of the known bacterial species, the uptake of DNA is random, whereas a minority display sequence preferences for uptake over the bacterial membrane (Thomas and Nielsen, 2005).

Natural transformation is the only one of the three HGT mechanisms that is initiated by the recipient cell. Not all cells in a bacterial population undergo natural transformation, only the proportion that is naturally competent.

Competence is a genetic encoded physiological state, characterized by the synthesis of specific competence proteins that enable bacteria to take up DNA through the cell membranes and wall (Claverys and Martin, 2003; Chen and Dubnau, 2004). Natural competence is described in at least 60 species of bacteria, such as *Streptococcus pneumoniae*, *Helicobacter pylori*, *Neisseria gonorrhoeae* and *Acinetobacter baylyi*. However, the proportion of species able to acquire DNA by natural transformation remains to be fully determined, as many species or strains may have the ability to perform natural transformation, without such capacity being searched or detectable under laboratory conditions. Laboratory techniques have limitations in sensitivity, time constraints and low representativeness regarding the environmental variables (Claverys and Martin, 2003; Johnsborg et al., 2007). The occurrence of naturally transformable species within the pool of uncultivable bacteria also remains to be fully explored. In addition, several species or strains, including *Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas stutzeri*, earlier considered as non-competent, have later been reported as naturally transformable under certain conditions (Bauer et al., 1999; Lorenz and Sikorski, 2000; Woegerbauer et al., 2002; Ramirez et al., 2010a). In a few species, such as *N. gonorrhoeae*, competence is constitutively expressed, while in the majority of the species competence is transient and usually dependent on growth-phase (Lorenz and Wackernagel, 1994; Johnsborg et al., 2007).

Natural transformation has been demonstrated in various bacterial species from a wide range of environments, such as soil (Chamier et al., 1993; Nielsen et al., 1997; Sikorski et al., 1998), water (Paul et al., 1991; Baur et al., 1996) and foodstuff (Bauer et al., 1999; Wittke et al., 2002).

Natural transformation can be divided into different steps, although the last two steps are not considered strictly part of the transformation mechanism (Thomas and Nielsen, 2005; Nielsen et al., 2009):

- 1) Presence of environmental factors leading to the development of genetic competence in the recipient bacterium while simultaneously being exposed to naked DNA;

- 2) Uptake of single-stranded DNA (ssDNA) in the recipient cytoplasm;
- 3) Heteroduplex formation of the transforming DNA with similar DNA sequences within the recipient chromosome (if exposed to chromosomal DNA), and integration of the DNA fragment into the recipient chromosome by homologous recombination;
- 4) Reconstitution of plasmids in the bacterial cytoplasm (if exposed to plasmid DNA);
- 5) Alteration of the bacterial phenotype as a consequence of expression of the acquired trait(s);
- 6) Vertical inheritance and maintenance of the acquired trait(s) at the individual and the population level.

3.1 *Acinetobacter baylyi*, a model for natural transformation

A. baylyi is a gram-negative, strictly aerobic, non-motile and versatile metabolic microorganism, found in soil and water. It was named *Acinetobacter calcoaceticus* for many years, until DNA-DNA hybridization studies allowed the establishment of a subdivision of the genus (Vaneechoutte et al., 2006). *A. baylyi* is a non-pathogenic bacterium (Barbe et al., 2004), though two cases of infection by this species have been reported in Asia (Chen et al., 2008; Zhou et al., 2011). *A. baylyi* is related with species of clinical importance (Bergogne-Berezin and Towner, 1996; Barbe et al., 2004).

UV mutagenesis of the mucoid *A. baylyi* strain BD4 resulted in a microencapsulated mutant that was named *A. baylyi* BD413, and later in the USA, *A. baylyi* ADP1. A main characteristic is that it does not aggregate in culture (Juni and Janik, 1969). The BD413 and ADP1 strains are assumed to be near-identical, although a few differences between the two laboratory strains are expected (Ornston et al., 2008). The ADP1 genome of 3.6 Mb has already been completely sequenced (Barbe et al., 2004).

The compact genome, the high level of competence for natural transformation, and the fast and easy laboratory growth, make *A. baylyi* an ideal model organism for genetic studies (Metzgar et al., 2004).

The development of competence in *A. baylyi* is growth-dependent, with maximum expression during the early exponential phase, where almost all cells in a culture express competence (Palmen and Hellingwerf, 1997). Fragments of both chromosome and plasmid DNA are taken up by this species, and uptake is indiscriminate with respect to the source (Palmen et al., 1993). *A. baylyi* grows optimally between 30 and 37 °C (Metzgar et al., 2004), and uptake of the DNA is maximal at 30 °C (Palmen and Hellingwerf, 1997). *A. baylyi* requires divalent cations for natural transformation, and EDTA inhibits transformation (Lorenz and Wackernagel, 1994). The strains are able to grow on both rich and minimal media (Metzgar et al., 2004).

A. baylyi initially binds double-stranded DNA (dsDNA), but such DNA enters the cell cytoplasm in a single-stranded form. The species has been reported to capture 60 basepairs (bp) per second (Palmen et al., 1993). In order to be maintained, the incoming DNA must be integrated into the chromosome of the recipient.

The most common method of DNA integration is through homologous recombination, a process that is catalysed by the RecA enzyme. Such recombination can be substitutive in cases of recombination between similar sequences, resulting in minor nucleotide changes. Homologous recombination can also be additive in cases of recombination with the recipient genome with a different donor sequence flanked by identical sequences to the recipient, resulting in additive integration of new DNA stretches. The latter form of recombination is common when the donor DNA is a circular molecule with limited stretches of sequence similarity (Thomas and Nielsen, 2005; Nielsen et al., 2009).

Another mode of recombination called homology-facilitated illegitimate recombination (HFIR), is based on that one end of the incoming DNA is homologous with the recipient DNA, and additional sequences are integrated due to the initial homology-based basepairing and a second basepairing occurring between microhomologies (3-8 bp) on the other end. HFIR can contribute to the acquisition of foreign DNA, though this is a rare process (de Vries and Wackernagel, 2002).

The uptake of a self-replicating plasmid is independent of homologous recombination but requires strand annealing and gap closure in the bacterial cytoplasm (Lorenz and Wackernagel, 1994; Nielsen et al., 1997; Thomas and Nielsen, 2005).

3.1.1 Intrinsic antibiotic resistance genes in *A. baylyi*

An *ampC* β -lactamase gene has been identified in the chromosome of *A. baylyi* ADP1, although this strain is susceptible to β -lactams. This can be explained by the absence of β -lactamase regulatory genes, such as the known promoter of *ampC*, the insertion sequence *ISAba1* (Heritier et al., 2006; Beceiro et al., 2007).

Resistance to eight antibiotics has been associated with 11 different genes present in the chromosome of *A. baylyi* ADP1: *acrB*, *oprM*, *ampD*, *mpl*, *pbpG*, *argH*, *hisF*, *gph*, *ACIAD0795*, *gshA*, and *recD*. The *gshA* and *recD* genes were specifically associated with decreased susceptibility to metronidazole and ciprofloxacin, respectively, whereas the other genes listed were connected to resistance to β -lactams (Gomez and Neyfakh, 2006).

3.2 Barriers to natural transformation

Although HGT is common among many species of bacteria, there are several limiting factors that affect the occurrence of gene exchange. Barriers to natural transformation can be divided into pre- and post-transformation barriers.

Pre-transformation barriers prevent the formation of transformed cells, and include the availability of DNA in the environment, the level of bacterial competence development, the activity of DNA restriction-modification systems, and the likelihood of genetic recombination caused by DNA sequence divergence. The pre-transformation barriers contribute to the sexual isolation of bacterial species and limit the exchange of genetic information between different bacterial species (Majewski, 2001). Post-transformation barriers

involve factors that affect the heritable stability of the acquired trait and survival of the transformed cell over time. The latter barrier represents the fitness cost imposed by the acquired DNA (Matic et al., 1996; Thomas and Nielsen, 2005).

The uptake of DNA is limited by several factors. Development of competence is linked to particular growth conditions and requires, in most cases, specific cellular and/or environmental signals; these requirements must be present, simultaneously with exposure with transforming DNA for natural transformation to occur (Thomas and Nielsen, 2005). The uptake of DNA can also be inhibited in the presence of chelating agents when this process requires cations; for instance, *A. baylyi* requires divalent cations for transformation and EDTA can block it (Lorenz and Wackernagel, 1994). The DNA released by dead or living bacteria may be degraded by DNases or can remain long enough in the environment to allow natural transformation (Nielsen et al., 2007). In fact, DNA has been found in different environments hours, days or even years, after its release (Wackernagel, 2006; Nielsen et al., 2007). In this way, DNA availability might not be a serious hindrance to natural transformation.

After DNA uptake, the DNA restriction-modification system and the degree of DNA similarity limit the integration of the incoming DNA into the host genome. The DNA restriction-modification systems protect the bacteria from infection by foreign DNA: the host restriction enzymes digest dsDNA that does not have the same sequence-specific chemical signatures, and modification enzymes modify the incoming DNA to allow restriction or the host DNA to protect it. However, the entrance of DNA as single-stranded in transformation confers some protection against this system (Matic et al., 1996; Majewski, 2001). The similarity between donor and recipient DNA sequences is essential for the activity of the enzyme responsible for the homologous recombination, RecA. Higher levels of sequence divergence leads to lower transformation frequencies (Matic et al., 1996). The requirements for high DNA similarity can be circumvented through illegitimate recombination, although this is a rare event (Majewski, 2001; de Vries and Wackernagel, 2002). Despite the high sequence specificity of the recombination enzyme in the initial stage, DNA regions with lower similarity can be integrated after the initiation of recombination at a MEPS (Minimum Efficient

Processing Segment) region, which represents the minimum amount of base-pairing required for the initial stage of the strand-exchange process. The MEPS for *A. baylyi* BD413 is 183 bp (de Vries and Wackernagel, 2002). Differences in the paired sequences after the initial stage of recombination are repaired by the mismatch-repair system (Matic et al., 1996). However, in transformable species this system seems to be a poor barrier to recombination (Majewski, 2001).

If novel genetic information of gene size or higher succeeds to be established in a cell, transcription and expression of the acquired genetic material may follow. Acquired genes conferring a fitness cost or other disadvantageous effects will concur against the stable acquisition and inheritance of such information (Majewski, 2001). On the other hand, the initial fitness costs imposed to the host can be reduced or lost through the occurrence of compensatory mutations and the acquired information may remain in the bacterial population (Wiedenbeck and Cohan, 2011).

4. Traits transferred by HGT

HGT contributes to the exchange of antibiotic resistance, virulence and metabolic traits (Ochman et al., 2000; Rankin et al., 2011). Antibiotic resistance genes are usually associated with MGEs, which increases their potential for dissemination (Ochman et al., 2000; Bennett, 2008). HGT of MGEs is one of the main mechanism responsible for the spread of antibiotic resistance genes (Aminov, 2011).

An example of movement of antibiotic resistance determinants by HGT includes the transfer of multidrug resistance plasmids from clinical strains of *E. coli* to *E. coli* HB 101 by conjugation; in this study, transfer of single and multiple plasmids was observed (Baral et al., 2012). Antibiotic-coding resistance genes plasmids were also shown to be transferred to *Vibrio* sp. DI-9 by natural transformation (Frischer et al., 1990).

MGEs are not limited to dissemination of resistance genes – they can also carry virulence (Hacker and Kaper, 2000) and metabolic genes (Springael and Top,

2004; Andam et al., 2011). For instance, phages have a huge potential for transfer of virulence factors (Hacker and Kaper, 2000). The transfer by transduction of the pathogenicity islands SaPIs, containing several toxin-coding genes, from *Staphylococcus aureus* to *Listeria monocytogenes* was demonstrated by Chen and Novick (Chen and Novick, 2009). Self-transfer by excision and reintegration of an ICE, the ICEclc, containing genes involved in metabolic functions, from *Pseudomonas knackmussii* to *Pseudomonas putida* and *Cupriavidus necator* has also been shown (Sentchilo et al., 2009).

4.1 Transfer of antibiotic resistance

Most antibiotic resistance traits can be transferred by HGT (van Hoek et al., 2011); whole genes or fragments of genes can be moved between species and change the antimicrobial susceptibility profiles of the recipient bacteria (Maiden, 1998). Resistance determinants that can be transferred by HGT include those coding for resistance to β -lactams, aminoglycoside-modifying enzymes, quinolone resistance genes, and sulphonamide resistance determinants.

Horizontally acquired resistance to β -lactams can be related with acquisition of a new β -lactamase, induction of expression of the β -lactamase AmpC, alterations in the expression of efflux pumps, and acquisition or alteration of penicillin binding proteins (PBPs) (Pfeifer et al., 2010). As alterations in PBPs were screened for in our study, more detailed information related with PBPs-associated resistance will follow.

PBPs

Acquisition of low-affinity PBPs, over-expression of an endogenous PBP or alteration of endogenous PBPs are the bacterial strategies related with PBPs for increased resistance to β -lactams (Zapun et al., 2008). For example, alterations in the PBPs lead to a decreased ability of the β -lactams to bind effectively, reducing disruption of cell wall synthesis.

Transfer of the *penA* gene, which codes for the PBP 2, was demonstrated to occur by natural transformation from *N. gonorrhoeae* NG0003 and ST7363 strains to NG0202 and ST1901 strains; a few point mutations were also detected in the transferred gene (Ohnishi et al., 2010). However, rather than be transferred as entire genes, endogenous PBPs tend to be altered due to the substitution of blocks of DNA from different conspecies, resulting in the formation of mosaic genes. Several reports on the formation of PBP mosaic genes as a result of HGT and recombination events can be found in the literature. For example, Lujan and colleagues (Lujan et al., 1991) found evidence that the resistance to penicillin shown by two isolates of *Neisseria lactamica* was due to the replacement, probably by transformation, of a region of 175 bp in the *penA* gene, from *Neisseria flavescens*. Natural transformation of the susceptible *H. pylori* 700392 with DNA from the β -lactams resistant *H. pylori* IH-1 resulted in transformants that acquired a mosaic block containing the C-terminal portion of the *pbp-1A* gene, which encodes the PBP 1A, and that resulted in changes in the antimicrobial susceptibility profiles (Kwon et al., 2003).

The same phenomenon of mosaic gene formation due to HGT can be seen for some cases of acquired sulphonamides resistance (Maiden, 1998). In this situation, mosaic alleles occur in the dihydropterate synthase gene, *dhps*. For instance, the comparison between four resistant and four susceptible strains of *Neisseria meningitidis* to sulphonamides, demonstrated the horizontal acquisition of DNA segments in the *dhps* gene of the resistant strains (Radstrom et al., 1992).

II. Fitness cost of antibiotic resistance genes

The biological cost conferred to the host by a resistance gene is considered a key parameter on the spread and stability of resistant bacteria (Andersson,

2003). The fitness burden imposed by antibiotic resistance genes in a bacterium is determined by the relative rates at which resistant and sensitive bacteria survive, or not, are transmitted between different cells, and are eliminated from infected hosts (Bjorkman and Andersson, 2000). In general, most studies show that initially transferred antibiotic resistance genes impose a fitness cost to the host bacterium (Andersson, 2003; Sandegren et al., 2008). However, the majority of the fitness studies report the biological cost associated with either chromosomal resistance mutants or plasmid-located resistance genes (Bjorkman and Andersson, 2000). Fewer studies have determined the cost of singly transferred resistance genes (Starikova et al., 2012).

The host fitness cost imposed by a resistance determinant would suggest that in the absence of the antibiotic, the resistance would be lost. However, bacteria can ameliorate the cost, due to compensatory mutations, allowing the persistence of the gene even in the absence of positive selection (Andersson and Hughes, 2010). Additionally, not all resistances confer a cost; they can be neutral (Enne et al., 2005; Pranting et al., 2008) or even enhance the fitness of the host (Luo et al., 2005; Rozen et al., 2007). Other selection on the host population can, in some cases, allow the maintenance of genes without positive host effects. For instance, selection of one resistance gene/trait will contribute to maintain adjacent and linked genes, even when those genes have no beneficial effect on host fitness (Barton, 2000; Kim and Stephan, 2002; Andersson, 2006).

Prohibiting the use of antibiotics is often considered a strategy to reduce antimicrobial resistance, with the assumption that resistance genes impose a fitness cost to the host and the absence of the antibiotic pressure will lead to their loss. However, reversibility of resistance is challenging, and it has been demonstrated that part of the bacterial population will maintain the acquired resistance traits even after the removal of antibiotic pressure (Johnsen et al., 2009; Andersson and Hughes, 2010).

III. Mobile genetic elements

MGEs are common elements in bacterial communities due to their capacity to physically move within and between host genomes. The abundance of MGEs in bacterial communities can be used as a measure of the HGT events that contribute to the bacterial evolution (Wright et al., 2008; Aminov, 2011). Conjugation, transduction and natural transformation contribute to the movement of genetic information between cells, while homologous or illegitimate recombination, transposition and site-specific recombination allow the intracellular movement of the DNA (Bennett, 1999; Toussaint and Merlin, 2002).

A variety of MGEs have been described to date, such as plasmids, bacteriophages, genomic islands (GIs), ICEs, insertion sequences (ISs), transposons (Tns), integrons and miniature inverted repeat transposable elements (MITEs). Often, several different MGEs are combined in one complex functional module (Rice, 2002; Toussaint and Merlin, 2002; Stokes and Gillings, 2011). The different types of MGEs are found both in Gram-negative and -positive bacteria.

The next sub-chapters will refer to MGEs other than integrons. As the integrons are the main focus of this study, they will be object of description in a separate section (IV).

1. Miniature inverted repeat transposable elements

MITEs are non-autonomous mobile elements consisting of small repeat sequences, which do not encode proteins and are found in random locations in the genome of various bacteria (Delihias, 2008, 2011). MITEs are quite abundant (Filee et al., 2007; Delihias, 2008; Han and Wessler, 2010) and their capacity of transposition has been shown in the genomes of archaea (Blount and Grogan, 2005), eukaryotes (Yang et al., 2009) and bacteria (Delihias, 2011). Bacterial MITEs do not carry a transposase gene, but are suggested to

transpose when a transposase is provided *in trans*. The movement of MITEs generates a target site duplication (TSD), which are short direct repeats on each side of the element. MITEs also possess terminal inverted repeats (IRs) typical of the ISs, which might indicate they were originated from these elements (Siguier et al., 2006; Delihias, 2011).

2. Insertion sequences

ISs are one of the most basic and smallest mobile elements, consisting of a central region encoding a transposase, responsible for mobility, flanked by terminal IRs; ISs only encode their own mobility. Transposition of ISs also produce TSD (Mahillon and Chandler, 1998). ISs are important for the dissemination of antibiotic resistance genes, and contribute to the shaping and reshuffling of the bacterial genome, as they often flank genes and are capable of moving neighbouring genes (Mahillon and Chandler, 1998; Siguier et al., 2006). Some ISs can also control the expression of adjacent genes (Mahillon and Chandler, 1998). A DNA segment flanked on each side by the same IS is called a composite transposon, where at least one of the two IS copies must be intact to confer mobility (Toleman et al., 2006b). There are about 20 known families of ISs, and they are present in most bacterial genomes (Siguier et al., 2006).

ISCRs (Insertion Sequence Common Regions) are an unusual group of IS that belong to the IS91 family and are characterized by the lack of the inverted repeats and by dissimilar ends, designated *ori*S (origin of replication) and *ter*S (termination site). ISCR also transposes but by a mechanism different from other ISs, called rolling-circle, and results in 'one-ended transposition'. In this type of transposition, the movement relies only on the function of one end. ISCRs do not generate TSD, which makes it difficult to identify DNA mobilised by this type of IS in sequencing analysis. A single copy is able to mobilise adjacent DNA (Tavakoli et al., 2000; Toleman et al., 2006b, 2006a).

3. Transposons

Tns are distinguished on the basis of intercellular transferability: some are mobilisable and other are conjugative (Roberts et al., 2008). Mobilisable Tns (MTns) do not encode functions for self-transfer, but can hitchhike on other MGEs to move. Conjugative Tns (CTns), also designated ICEs, have different characteristics from MTn and will be described in the next subsection (4). Conjugative and non-conjugative Tns have also different methods of transposition (Salyers et al., 1995).

MTns have a wide range of sizes and are characterized by the presence of IRs on both ends; some MTns are formed by two ISs that flank a central region (Lupski, 1987). Transposition relies on the action of a transposase, usually part of the MTn, which recognizes the IRs of the MTn. The insertion of a MTn in a new location also generates TSDs, and can cause insertions, deletions and translocations of DNA (Lupski, 1987). Transposition does not require DNA homology between the element and the site of insertion; some Tns have specific nucleotide sequences as preferred insertion site (Bennett, 2008).

4. Integrative and conjugative elements

ICEs are self-transmissible elements that show characteristics of plasmids, phages and transposons; they have the ability to excise from the chromosome where they are located, disseminate to other cells by conjugation and integrate and replicate in the genome of the new host, though cannot replicate independently (Burrus and Waldor, 2004; Taviani et al., 2009). ICEs insert into a single site in the chromosome of the target, usually in well-conserved genes (Toleman and Walsh, 2011); they are thought to promote the mobilization of GIs (Burrus and Waldor, 2004). Unlike MTns, ICEs do not generate TSD when inserted in a new genome (Scott, 1992); so acquisition of these elements by HGT might be only inferred when comparing their nucleotide composition with the ancestral or core genome. The backbone of ICEs, containing genes

essential for their maintenance, dissemination and regulation, can acquire a big variety of genes, coding for a set of diverse functions, such as resistance and virulence (Burrus and Waldor, 2004).

5. Genomic islands

GIs are large chromosomal regions containing genes encoding a diverse range of functions, which show evidence of acquisition by HGT, such as a different GC content and codon usage from the core genome. When these islands encode resistance to antibiotics and heavy metals, they are denominated resistance islands; pathogenicity islands encode virulence factors; metabolic islands provide the ability to utilize novel carbon and nitrogen sources; and degradation islands give the ability to break down novel compounds (Boyd et al., 2009). Similar GIs are usually inserted in defined sites on the chromosome of different host chromosomes (Boyd et al., 2009; Krizova and Nemeč, 2010). Most of the GIs are not self-mobile and transfer of GIs is supposed to occur by hitchhiking with other MGEs (Boyd et al., 2009; Waldor, 2010); however, recently, transfer of a few GIs has been demonstrated (Juhas et al., 2008).

6. Bacteriophages

Bacteriophages are abundant forms of life and co-reside in environments with bacteria (Hanlon, 2007), which are confronted with a constant threat of phage infection (Stern and Sorek, 2011). Bacteriophages can be divided in virulent and temperate phages: virulent phages promote the lysis of the host cell (lytic cycle); temperate phages can integrate in the chromosome of the host and replicate with the bacteria (lysogenic cycle), though they can also undergo the lytic cycle (Frost et al., 2005; Rankin et al., 2011). The genome of bacteriophages can be composed by ss or dsDNA or RNA, and have a wide range of sizes (Frost et al., 2005); it can exist in the host cell either in an extrachromosomal form or integrated in the host DNA (prophage). Phages

genome can be integrated in the host DNA by transposition or by site-specific recombination (Campbell, 1992), and can also transfer fragments of DNA from one to another host (Frost et al., 2005).

7. Plasmids

Plasmids are extrachromosomal DNA molecules, typically circular and double-stranded, which replicate independently of the chromosome; when integrated in the chromosome, they are called episome. Plasmids are conjugative, when they encode the functions needed for self-mobilization, or mobilisable, when they rely on the help of conjugative elements to move between cells (Bennett, 2008). These MGEs have a wide range of sizes, which usually depend on their mobility mechanism; conjugative plasmids are much bigger than mobilisable ones (Bennett, 2008). Some plasmids can have the size of small chromosomes and are called megaplasmids (Frost et al., 2005). Plasmids do not usually contain genes needed for essential cellular functions, but contain a backbone of genes encoding plasmid replicative and transfer functions, and often a variety of additional genes including antimicrobial resistance and virulence determinants (Frost et al., 2005; Rankin et al., 2011). Plasmids are ubiquitous and they have been identified in most bacterial species investigated; carriage of multiple plasmids is also common (Bennett, 2008).

IV. Integrons

Integrons are genetic elements that can integrate and excise genes by site-specific recombination, and are also responsible for their expression. They were initially described at the end of the 1980s (Stokes and Hall, 1989). Bioinformatics based analysis of partially or fully sequenced bacterial genomes show that integrons or integrase genes are present in approx. 10 % and 17 % of

them, respectively (Boucher et al., 2007; Cambray et al., 2010). Most of the descriptions of integrons are done in human clinical isolates (Levesque et al., 1995; Gombac et al., 2002; Cambray et al., 2010), but they are also found in many non-clinical environments, such as in aquatic environment (Rosser and Young, 1999; Moura et al., 2007; Wright et al., 2008), soil (Nield et al., 2001; Srinivasan et al., 2008), food (Antunes et al., 2004; Van et al., 2012) and food-producing animals (Goldstein et al., 2001; Van et al., 2012).

Integrons consist of three elements: the gene that encodes the integrase (*intI*), needed for site-specific recombination within the integron; the adjacent recombination site (*attI*) that is recognized by the integrase; and the promoter (P_c), located upstream of the integration site, necessary for efficient transcription and expression of gene cassettes present in the integron. Although the P_c promoter is assumed to be part of all classes of integrons, its presence and activity has not been shown for all of them (Boucher et al., 2007). Integrons can incorporate one or more gene cassettes (Hall and Collis, 1995). Numerous combinations of gene cassettes have been reported so far (Partridge et al., 2009).

1. Classification of integrons

1.1 Genomic context

Integrons classification based on their genomic context includes two different types of integrons, mobile integrons (MIs) and chromosomal integrons (CIs) (Cambray et al., 2010). CIs can carry a variable number of gene cassettes, ranging from zero to hundreds, which are usually not involved in antimicrobial resistance, though some carry antibiotic resistance genes. CIs are considered to be sedentary, though movement of gene cassettes from CIs has been reported, and are not associated with MGEs (Rowe-Magnus et al., 2001; Boucher et al., 2007; Cambray et al., 2010). CIs with a high number of gene cassettes (> 20) are denominated superintegrons (SIs) (Cambray et al., 2010).

SIs were first identified in *Vibrio cholerae* (Mazel *et al.*, 1998), but are present in more species and genera (Rowe-Magnus *et al.*, 2001; Fluit and Schmitz, 2004). In contrast, MIs carry a limited number of gene cassettes and are often involved in antimicrobial resistance dissemination (Cambray *et al.*, 2010). MIs are designated mobile, not because they have the capacity to move themselves, but because they are usually inserted into other MGEs with self-mobility capacity; and they can also be located in the chromosome (Mazel, 2006). MIs can capture some of the gene cassettes from CIs (Rowe-Magnus *et al.*, 2002).

1.2 Integrase nucleotide sequence

Integrans are also classified based on the genetic relatedness of the integrase *intI* gene sequence; different classes have an integrase gene with a nucleotide sequence identity inferior to 98 %. The nucleotide differences in integrases belonging to the same class are usually located in the promoter region (Stokes and Hall, 1989). The naming of integrases is confusing, as the same designation has been attributed to different integrases (Fluit and Schmitz, 2004). The *attI* site is also unique for each class, with the exception of a core site that conforms to the same consensus, GTTRRRY (where R = purine and Y = pyrimidine) (Nield *et al.*, 2001).

Class 1 integrans were the first described (Stokes and Hall, 1989), and have been reported as the most common and widespread, especially in clinical settings. They are found in many different Gram-negative and -positive genera. Integrans belonging to class 1 are commonly associated with transposons. This class of integrans is considered the main contributor to the problem of antibiotic resistance (Cambray *et al.*, 2010).

Most of the class 2 integrans are associated with transposons of the family Tn7; the *intI2* has an internal stop codon that results in a defective integrase (Hansson *et al.*, 2002). The gene cassettes found in this class are the same as the detected in class 1 integrans (Hansson *et al.*, 2002; Ramirez *et al.*, 2010b); and they are mainly found in bacteria of clinical origin (Ramirez *et al.*, 2010b).

Class 3 integrons are less prevalent than the two previous classes (Partridge et al., 2009), but have a similar structure (Arakawa et al., 1995; Collis et al., 2002; Correia et al., 2003).

Most of the gene cassettes carried in class 1, 2 and 3 integrons code for antibiotic resistance. Usually these integrons carry less than five cassettes (Bennett, 2008), although class 1 integrons with up to nine antibiotic resistance genes have been described (Naas et al., 2001).

The fourth class of integrons was first attributed to an integron in *V. cholerae* that differs from the previous ones because it carries a high number of gene cassettes (at least 179), and it has been named SI (Mazel *et al.*, 1998). Most of the gene cassettes in SIs code proteins of unknown function; few result in resistance to antibiotics, while others have virulent and metabolic functions (Rowe-Magnus et al., 2001; Rowe-Magnus et al., 2003). The class 4 was also later attributed to an integron, containing five gene cassettes, present in an ICE, designated SXT, on a *V. cholerae* chromosome (Hochhut et al., 2001).

Class 5 integron was originally attributed to a SI identified in a *Vibrio mimicus* isolate as well (Clark et al., 2000). The same class 5 was also attributed to the integron carried in the plasmid pRVS1 of *Vibrio salmonicida* (Sorum et al., 1992; Boucher et al., 2006).

Nowadays, the SI designation is applied to large chromosomally-located integrons, rather than the numbered classes (Fluit and Schmitz, 2004).

Three other classes, classes 6, 7 and 8, were identified in environmental bacterial isolates and are not associated with antibiotic resistance; two of the classes possessed putative gene cassettes with unknown functions (Nield et al., 2001).

1.3 Phylogeny of the integron integrase gene

A recent extensive DNA sequencing effort has revealed more than 90 different classes of integrons, based on the phylogeny of the integrase gene; most of these classes include CIs (Boucher et al., 2007; Gillings et al., 2008). This classification defines three broad groups, the soil/freshwater proteobacteria, the

marine γ -proteobacteria, and the inverted integrase group (Boucher et al., 2007). The phylogenetic tree of the integron integrase reflects the occurrence of HGT of integrons.

This work is focused on mobile class 1 integrons, and the characteristics of this class will be further developed.

2. Class 1 integrons

Usually, class 1 integrons have three distinct genetic regions: two are highly conserved, the 5'-conserved segment (5'-CS) and the 3'-conserved segment (3'-CS), and flank the central variable region where the gene cassettes are located (Stokes and Hall, 1989). The 5'-CS includes the integrase *intl1* gene, the *attI1* site and the promoters P_c (previously called P_{ANT}), and P_2 when present. The 3'-CS consists of the *qacE Δ 1* gene, which encodes an incomplete version of a protein that mediates resistance to certain detergents, the *sulI* gene, encoding resistance to sulphonamides, and an open reading frame, *orf5*, of unknown function (Figure 2) (Fluit and Schmitz, 1999). The central variable region can have different numbers of gene cassettes; gene cassettes can also be absent. The simplest integron, In_0 , does not have any gene cassette; different gene cassettes and different numbers of cassettes can be captured, resulting in integrons with different gene cassette arrays (Figure 3) (Bennett, 1999). Each cassette array gives a number to the integron; a list of numbered class 1 integrons is available from the INTEGRALL database (<http://integrall.bio.ua.pt/>) (Moura et al., 2009) – so far there are about 800 different gene cassette arrays (accessed on November 2012).

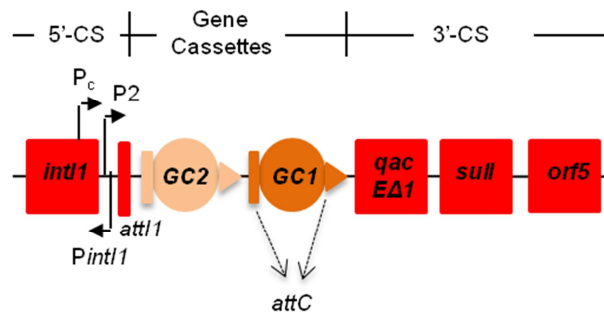


Figure 2. Schematic structure of a class 1 integron. *int1* – class 1 integrase gene; *Pint1* – integrase promoter; P_c and P_2 – gene cassettes promoters; *att1* – integron-associated recombination site; *qacE Δ 1* – truncated version of a quaternary ammonium resistance gene; *sull* – sulphonamide resistance gene; *orf5* – open reading frame; *attC* – recombination site of the gene cassette; GC – gene cassette; 5'-CS – 5' conserved segment of the integron; 3'-CS – 3' conserved segment of the integron.

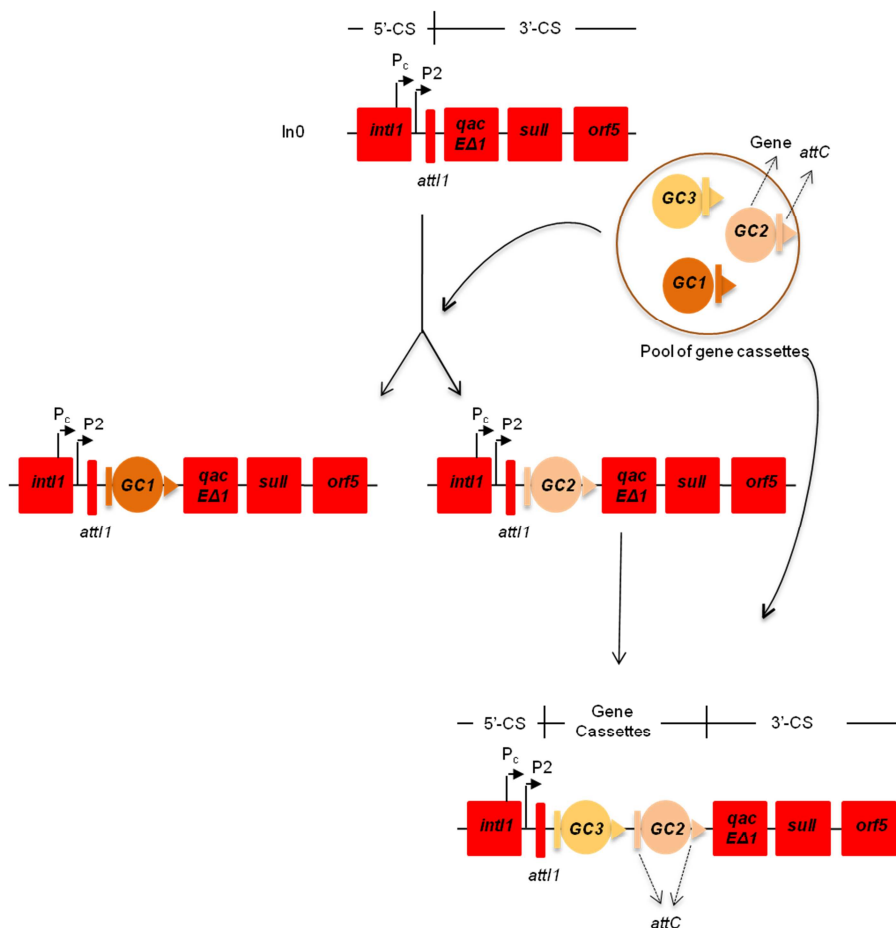


Figure 3. Schematic representation of the acquisition of gene cassettes by a class 1 integron. In0 does not contain any gene cassettes. Different cassette arrays can be acquired and be part of the variable region of the integron.

Although most of the class 1 integrons have the classical structure described above, an increasing number of complex class 1 integrons have been reported (Toleman et al., 2006b; Quiroga et al., 2007; Song et al., 2010). These non-classic class 1 integrons probably originated from the association of *ISCR1* with classic class 1 integrons, leading to the deletion of part of the 3'-CS (Toleman et al., 2006a). Complex class 1 integrons contain usually a typical class 1 integron (with truncated 3'-CS), followed by an *ISCR1*, another variable region and another 3'-CS; an additional *ISCR1* element can follow. In the second variable region, non-cassette type resistance genes are usually present (Toleman et al., 2006b, 2006a; Bennett, 2008).

2.1 Integrase

The integrase of the integron belongs to the tyrosine recombinase family and is responsible for site-specific recombination, which involves reciprocal exchange between defined DNA sites. The *IntI* promotes the integration and excision of gene cassettes in integrons (Grindley et al., 2006). Site-specific recombination requires limited homology between the short recombination DNA specific sequences (Gravel et al., 1998). In addition to the two conserved regions, box 1 and box 2, and three shorter conserved regions designated patch I, II and III, typical of the tyrosine recombinases, the integrases have an insertion of 16 amino acids between patch II and III (Nield et al., 2001).

Ten different variants of the class 1 integrase *IntI1*, resulting from 13 different variants of the gene cassette promoter P_c , have been identified (Jove et al., 2010). The *intI1* codes a protein with 337 amino acids (Stokes and Hall, 1989).

2.1.1 Integrase SOS regulation

Recent publications demonstrate that the integrase genes in integrons carry LexA binding sites in the vicinity of their promoter regions and can be controlled by the host LexA protein, which is a transcriptional repressor of the SOS

response. This observation was verified for the class 1 integrase *intI1* and for the integrase *intIA* present in a CI of *V. cholerae* (Guerin et al., 2009; Baharoglu et al., 2010). Therefore, in organisms harbouring *lexA* alleles, SOS induction can lead to increased transcription of the integrase gene and increased integrase activity, including cassette rearrangements (Guerin et al., 2009; Baharoglu et al., 2010). *In vivo* rearrangement of gene cassettes due to induced class 1 integrase expression after triggered SOS response has also been observed (Hocquet et al., 2012).

SOS induction of the expression of the integrase by conjugation (Baharoglu et al., 2010) and natural transformation (Baharoglu et al., 2012) has been recently shown in *V. cholerae* and *E. coli*, and in *V. cholerae* only, respectively. The regulation of the integrase expression in species where a LexA orthologue is absent, such as in *A. baylyi* (Hare et al., 2006), is unknown.

2.2 Integron promoter

The integrase gene *intI1* is expressed from the promoter *PintI1*; *PintI1* and P_c are convergent, and *PintI1* and P2 overlap (Levesque et al., 1994).

The relationship between expression of the gene cassettes and the integrase is not clear, as two different studies have opposite conclusions. According to Guérin and collaborators (Guerin et al., 2011), expression of gene cassettes and integrase is highly connected; P_c expression interferes with expression of *PintI1*, while P2 does not have any influence in the transcription of the integrase, though the P2 disrupts the LexA binding site. However, the influence of the P_c depends on the variant; only the P_cS inhibits the *PintI1* expression. In contrast with these results, Wei and collaborators (Wei et al., 2011) showed that neither the P_c , regardless the variant, nor the P2 influenced the integrase gene expression. Guérin *et al.* suggested that the different results are due to the fact that the *PintI1* was repressed in the Wei *et al.* study (Guerin et al., 2011). In a recent study, an inverse correlation between the strength of the P_c promoter and the integrase activity is suggested (Starikova et al., 2012).

2.3 Gene cassettes

Gene cassettes are small mobile elements composed by a single and often promoterless gene, and a recombination site (*attC*). The size of the different gene cassettes vary considerably, and the difference is mainly due to the size of the gene (Recchia and Hall, 1995). Gene cassettes are linear when integrated in the integron and circular in the free form, after excision or before site-specific insertion (Collis and Hall, 1992a); free circular cassettes are not able to replicate (Hall and Collis, 1995). The *attC* site is recognized by the integrase and recombination between this site and the *attI1* site of the integron backbone leads to the addition of the gene cassette into the integron structure. A huge number of cassette arrays have been reported (Partridge et al., 2009). The majority of the 194 known gene cassettes (< 98 % identity) encode antibiotic resistance against antibiotics in use for long time but also newer introduced ones (132 against the 62 coding unknown functions); including resistance to frequently used aminoglycosides, β -lactams, quinolones, chloramphenicol and trimethoprim (Partridge et al., 2009). Gene cassettes are identified by the name of the gene that they encode or by *orf* or *gcu* when the function is unknown (Recchia and Hall, 1995; Partridge et al., 2009). Resistance to the same antibiotic can be conferred by several different gene cassettes, sometimes with a different mechanism of action (Recchia and Hall, 1995).

The *attC* sites, previously called 59-base elements or 59-be because of the size of the first identified recombination sites, of the diverse gene cassettes vary significantly in length and sequence, but share a common feature, the two imperfect inverted repeats with two core regions. On the 3' end of the gene is the consensus RYYAAC and on the other end the GTTRRRY (this consensus is identical with the one present on *attI1* site). Due to the physical nature of the recombination event, part of the *attC* site is located in the start of the gene and part in the end (Recchia and Hall, 1995; Fluit and Schmitz, 1999).

Transcription and posterior expression of gene cassettes depends on the promoter of the integron, as the gene cassettes themselves rarely possess a promoter (Hall and Collis, 1995), though a few exceptions have been reported

(Partridge et al., 2009). The cassette must be inserted in the right orientation in order to be expressed from the promoter (Recchia and Hall, 1995). The level of transcription and expression of the gene cassettes depends on a few factors, such as the strength of the promoter, distance of the gene from the promoter, the presence of an additional internal promoter, and the number of copies of each gene (Levesque et al., 1994).

2.3.1 Gene cassette promoter

All gene cassettes are co-transcribed from the same promoter (P_c). The level of expression of the gene cassettes depends on the promoter strength (Jove et al., 2010). Expression is also influenced by the distance to the promoter, where cassettes closer to the promoter are expressed at higher levels than more distal ones (Collis and Hall, 1995; Rowe-Magnus et al., 2002); distal cassettes can change position and move closer to the promoter (Collis and Hall, 1992b; Rowe-Magnus et al., 2002; Baharoglu et al., 2010). The same cassette can have different expressions levels depending on the promoter and on the array where it is located.

The P_c promoter is located within the integrase coding sequence, but it is divergent to the *int1* gene. Thirteen different variants of the gene cassette promoter P_c , with different expression levels, have been described, based on the -35 and -10 hexamer sequences and the sequence upstream the -10 box (creating an extended -10 promoter) (Jove et al., 2010). The P_c can be associated with a second gene cassette promoter, P2 (Collis and Hall, 1995). Four variants of P2 have been identified (Jove et al., 2010; Vinue et al., 2011). P_cW (Weak) is the most prevalent of the P_c variants (Jove et al., 2010; Vinue et al., 2011). From the 13 P_c variants, four are associated with the only active form of P2. The P2 is mainly associated with P_cW , being, in this situation, the main responsible of the combination for the expression of the gene cassettes. The P_cS (Strong) is mostly linked with the inactive form of P2 (Levesque et al., 1994; Collis and Hall, 1995; Jove et al., 2010). A nucleotide space of 17 bp is associated with highest efficiency of the expression provided by the promoters;

a space of 14 bp between the pre-existing -35 and -10 boxes renders a non-functional P2 promoter (Levesque et al., 1994; Collis and Hall, 1995). A TGN-10 motif upstream of the -10 box of P_c , usually the variant P_cW , increases the efficiency of this promoter (Jove et al., 2010).

2.4 Recombination of gene cassettes

Recombination catalysed by the IntI1 occurs mainly between the two recombination sites *attI1* and *attC* of the integrase and the gene cassette, respectively. The integrase can also catalyse recombination between two *attI1* or two *attC* sites. In all cases, the recombination crossover occurs between the G and TT of the consensus GTTRRRY (Collis et al., 2001). The integrase IntI1 catalyse the excision of gene cassettes as well (Collis and Hall, 1992b).

As the recombination between the *attI1* and *attC* sites is preferred, newly acquired gene cassettes are usually inserted at the first position in the cassette array (Collis et al., 2001; Cambray et al., 2010).

Recombination between two *attC* sites can lead to the integration of the gene cassette at any position of the array; when the two *attC* sites are located in the same cassette array, recombination leads to the excision of a circular cassette intermediate (Cambray et al., 2010).

Recombination between two *attI1* sites can also occur, but it is quite inefficient (Collis et al., 2001).

Integrase activity can also lead to the integration of gene cassettes at (non-specific) secondary sites, containing a GNT consensus, though this occurs at very low frequency (Recchia et al., 1994). However, the excision of gene cassettes from the secondary sites is unlikely to happen, as the cassettes are not flanked by two active recombination sites (Hall and Collis, 1995). In these cases, expression of the cassettes depends on the presence of a promoter close to the integration site (Hall and Collis, 1995).

2.5 Molecular epidemiology of class 1 integrons

Class 1 integrons are mainly found in Gram-negative bacteria, though several studies have reported the presence of these mobile elements also in Gram-positive bacteria. In fact, Nandi and collaborators (Nandi et al., 2004) suggested that Gram-positives represent a larger reservoir of class 1 integrons than Gram-negatives. Reports of class 1 integrons in the Gram-positive genera *Corynebacterium* (Nesvera et al., 1998; Nandi et al., 2004), *Enterococcus* (Shi et al., 2006), *Staphylococcus* (Shi et al., 2006; Xu et al., 2008b; Xu et al., 2011a) and *Streptococcus* (Shi et al., 2006) are available. Nonetheless, the majority of the reports of class 1 integrons concerns Gram-negative genera, such as *Acinetobacter* (Gu et al., 2007; Xu et al., 2009), *Aeromonas* (Moura et al., 2007), *Burkholderia* (Crowley et al., 2002), *Enterobacter* (Xu et al., 2009), *Escherichia* (White et al., 2001; Moura et al., 2007; Xu et al., 2009), *Klebsiella* (White et al., 2001; Xu et al., 2009), *Morganella* (Moura et al., 2007), *Proteus* (White et al., 2001), *Pseudomonas* (Gu et al., 2007; Xu et al., 2009), *Salmonella* (Antunes et al., 2006; Krauland et al., 2009), and *Vibrio* (Falbo et al., 1999).

Integron-carrying Gram-positive bacteria have mainly been isolated from human clinical samples, while integron-carrying Gram-negative isolates have been collected from a wider range of environments, including hospitals (Levesque et al., 1995; Gombac et al., 2002; Antunes et al., 2004; Kang et al., 2005), healthy humans (Kang et al., 2005; Sepp et al., 2009), animals (Kang et al., 2005; Vo et al., 2007a; Machado et al., 2008), food-products (Antunes et al., 2004; Meng et al., 2011), soil (Agero and Sandvang, 2005; Srinivasan et al., 2008) and aquatic environments (Rosser and Young, 1999; Moura et al., 2007; Wright et al., 2008).

Class 1 integrons have been reported around the world, mainly from Europe and Asia, but also from other continents (Fluit and Schmitz, 1999, 2004). European reports include high incidence of this class of integrons. Forty-three per cent (70/163) of unrelated Gram-negative isolates (16 different species) obtained from clinical environments in nine European countries (Austria, Belgium, France, Germany, Italy, Poland, Portugal, Spain and UK) carried class

1 integrons (Martinez-Freijo et al., 1998); 44 % (16/36) of unrelated clinical *A. baumannii* isolates from Italy contained class 1 integrons (Gombac et al., 2002); 185 Gram-negative (four genera) and 72 Gram-positive (two genera) isolates collected from Danish farmland and pigsties were screened for class 1 integron presence, which showed to be present in 25 of the isolates (9.7 %) (Agero and Sandvang, 2005). Asian studies reported also a high prevalence of class 1 integrons. In Korea, among 664 *E. coli* isolates studied, class 1 integrons were detected in 30.8 % (62/201) of the human clinical isolates, 11.4 % (19/167) of the healthy human isolates, 42.3 % (69/163) of commensal poultry isolates and 18.8 % (25/133) of the commensal swine isolates (Kang et al., 2005); from 232 Gram-negative isolates collected from zoo animals in Japan, 6.9 % (16), belonging to six different species, carried a class 1 integron (Ahmed et al., 2007b); 28 % (belonging to 11 different serovars) of 297 unrelated non-typhoid *Salmonella* isolates (38 serovars) collected from clinical human samples (n = 56) and from healthy and sick cattle (n = 63), pigs (n = 111), and poultry (n = 67), in Vietnam were detected to carry different class 1 integrons (Vo et al., 2010). All the 37 *Aeromonas* spp. (six species) isolated from South African aquaculture systems carried at least one class 1 integron (Jacobs and Chenia, 2007); 53.1 % (52) of the 98 epidemiologically unrelated *Salmonella* isolates, representing 13 serovars, collected from slaughter animals and food products of animal origin in Ethiopia, contained class 1 integrons (Molla et al., 2007). Reports from America include, for example, the detection of class 1 integrons in 11.4 % (14/123) of the clinical *Acinetobacter* spp. isolates (at least five species were represented in the samples, but all positives were *A. baumannii*) obtained from inmates of 20 California correctional facilities (Golanbar et al., 2011); and the presence of class 1 integrons in 40.7 % (55/135) of the *Salmonella enterica* isolates (positive isolates belonged to 17 different serovars) collected from humans (n = 48), animal feed (n = 41), food-producing animals (n = 23), foodstuff (n = 11) and other sources (n = 12) from different Brazilian regions (Peirano et al., 2006). A study conducted in Australia detected 58 class 1 integrons among 54 of the 120 (45 %) *Enterobacteriaceae* (four genera) isolated from clinical samples (White et al., 2001).

Instead of checking for the presence of class 1 integrons, some studies look for the presence of class 1 integrases; this can contribute to detection of complex class 1 integrons or integrons with different structures (Post et al., 2007; Betteridge et al., 2011), that would not be detected with primers for the conserved regions of classical class 1 integrons. On the other hand, presence of class 1 integrase does not always correspond to the presence of a class 1 integron (Barlow et al., 2004; Golanbar et al., 2011).

Many of the studies that screen for the presence of class 1 integrons also check for class 2 integrons, and sometimes class 3 as well. Usually class 1 is present in a higher proportion of the isolates (White et al., 2001; Kang et al., 2005; Peirano et al., 2006; Ahmed et al., 2007b); some of the isolates contain both classes 1 and 2 (White et al., 2001; Kang et al., 2005; Dubois et al., 2007); presence of class 3 integrons is often negative (White et al., 2001; Kang et al., 2005; Dubois et al., 2007). The same isolate can also carry multiple class 1 integrons (Martinez-Freijo et al., 1998; White et al., 2001; Molla et al., 2007).

A few studies analysed and screened the presence of class 1 integrons worldwide. For instance, Seward and Towner (Seward and Towner, 1999) examined the presence of integrons in 25 nosocomial isolates of *Acinetobacter* spp. collected in 11 different countries (Argentina, Australia, England, France, Germany, Italy, South Africa, Singapore, Spain, The Netherlands and Wales); class 1 integrons were detected in 68 % (17) of the isolates. Another study, conducted with 68 *Shigella* spp. strains (four different species) collected in a French Hospital from patients that had been recently travelling to Africa, Asia, America and other countries in Europe, detected class 1 integrons in 32 (47 %) of the isolates (Dubois et al., 2007).

Integron-carrying strains are, usually, more resistant to antimicrobials than non-carrying integron ones (Martinez-Freijo et al., 1998; Severino and Magalhaes, 2002; Leverstein-van Hall et al., 2003).

Integron-carrying strains have also been involved in outbreaks: five *A. baumannii* isolated from a hospital in Australia contained the same class 1 integron (Mak et al., 2009); among 41 *Enterobacteriaceae* (four species) involved in an outbreak in a Spanish hospital, 20 isolates contained one class 1

integron and 21 another class 1 integron (Tato et al., 2010); all the 32 *V. cholerae* collected from an outbreak in Albania and Italy contained a class 1 integron with the same gene cassette array (Falbo et al., 1999). The same integron associated with outbreaks is also found in sporadic cases (Falbo et al., 1999; Mak et al., 2009).

2.6 Origin of class 1 integrons

The origin of class 1 integrons is not clear, but it is assumed that they were present in bacteria before the “antibiotic era” (Stokes et al., 2006). Class 1 integrons without antibiotic resistance genes have been found in environmental *Betaproteobacteria* that have been suggested to be the original source of these elements. These integrons contained the *intl1* gene, the *attI1* site and the P_c promoter, but lacked the Tn402 features (Stokes et al., 2006; Gillings et al., 2008). It is proposed that these class 1 integrons were incorporated into a plasmid-borne Tn402 transposon, probably carrying a gene cassette that conferred advantage (Gillings et al., 2008). This gene could be the *qac* gene, which codes resistance to quaternary ammonium compounds, as biocides have a long history of use in clinical practices (Gillings et al., 2009b), and also the *sull* gene, given that sulphonamides were one of the first antibiotics introduced in the clinical practice (Toleman and Walsh, 2011).

Another possibility for the class 1 integrons origin results from the association between the ancestor of the Tn402 transposon with a class 1 integrase and an *attI1* site; this is supported by the fact that most of the integrons contain the 5'-CS region at the same location (Toleman and Walsh, 2011). The Tn402 transposon is bounded by 25 bp IRs, contains the 5'-CS and a complete version of the *qacE* gene, but does not include the *sull* gene; also contains four genes that belong to the *tni* module (*tniR*, *tniQ*, *tniB* and *tniA*) (Radstrom et al., 1994). The 3'-CS region was formed by the fusion of the *qacE* gene with the *sull* gene, with consequent partial deletion of the *qacE* gene; this occurred at the same time as a deletion event in the transposition functions of the Tn402 transposon, resulting in a structure unable of self-mobilization (Toleman and

Walsh, 2011). Most of the class 1 integrons are defective transposons (Brown et al., 1996).

In0, which does not contain any gene cassette in the variable region, was first identified in the pSV1 plasmid of *Pseudomonas aeruginosa* strain PA025, and has also been suggested to be the ancestor of class 1 integrons (Bissonnette and Roy, 1992). In this study, it is suggested a common origin of the *intI1* and the *sull*, due to the similar codon usage of both genes. A probable combination between In0 and Tn402 would lead to the class 1 integron structure known nowadays, with a truncated version of the *qacE* gene, due to insertion of a sulphonamide resistance gene, and loss of two of the transposition genes (*tniR* and *tniQ*) and partial deletion of the *tniB*. At the same time, in opposition to this theory, there is some evidence showing that different integrons can have different origins (Partridge et al., 2001a).

After the formation of the conserved regions of class 1 integrons, strong selection for resistance to antimicrobials has favoured events of capture of antibiotic resistance gene cassettes into integrons, from the pool present in the microbial communities and in the environment (Bennett, 2008; Stokes and Gillings, 2011). There is also evidence that class 1 integrons can capture gene cassettes from SIs (Rowe-Magnus et al., 2002).

V. The genomic locations of class 1 integrons, context and mobility

Most of the descriptive studies of integrons focus on the identification and characterization of class 1 integrons and their gene cassette arrays; fewer studies aim to unravel the genetic context where they are inserted (Tato et al., 2010; Meng et al., 2011). When investigated, most of the integrons are found genetically linked to and embedded in MGEs, such as MITEs, ISs, Tns, GIs, and plasmids; some of these MGEs contain self-mobility determinants, whereas others can only be mobilised by other elements or when some genes/elements

are provided *in trans*. These elements confer mobility to the integrons when part of the larger MGE. Thus, genetic linkage to various MGEs facilitates both intra- and intergenomic transfers at frequencies determined by the mobility functions and transfer dynamics of the elements.

1. Genetic linkage to MGEs

The genetic linkage between class 1 integrons and MGEs increases the potential of class 1 integrons for dissemination by HGT. Class 1 integrons are frequently observed in larger MGEs with several layers of horizontal mobility, and such combination of MGEs confers a broad horizontal dissemination potential to class 1 integrons. Thus, the mobility of class 1 integrons embedded in MGEs emerges mainly from a complex interplay between various modes of transposition and plasmid-based conjugative processes. The fate of newly transferred integrons is further modulated by functional and selective constraints to the stable maintenance of the elements in new hosts. MGEs such as plasmids can contribute to the transfer of class 1 integrons between cells, whereas GIs, MITEs, ISs and MTNs allow intracellular transfer and the integration of the class 1 integrons in the genome of the host. Some phages, ICEs and some GIs can have both functions.

Some examples of association between class 1 integrons and the diverse MGEs will follow, including those that reflect the combination of diverse MGEs. Table 1 shows some examples of the diverse MGEs associated with class 1 integrons.

Table 1. Examples of mobile genetic elements (MGEs) associated with class 1 integrons.

MGE	Species	Source of isolation	Country of isolation	Reference
MITE				
MITE-like	<i>Acinetobacter johnsonii</i>	Prawn	Australia	(Gillings et al., 2009a)
IMU	<i>Enterobacter cloacae</i>	Clinical	Canada	(Poirel et al., 2009)
IS				
IS6 family	<i>Escherichia coli</i>	Clinical	Poland	(Zienkiewicz et al., 2007)
	<i>Salmonella enterica</i> serovar Newport	Clinical	France	(Doublet et al., 2009)
	<i>Escherichia coli</i>	Clinical	France	(Naas et al., 2001)
	<i>Escherichia coli</i>	Clinical	Greece	(Miriagou et al., 2005)
	<i>Salmonella enterica</i> serovar Typhimurium	Clinical	Albania	(Miriagou et al., 2005)
ISCR1	<i>Aeromonas punctata</i>	Wastewater	China	(Xia et al., 2010)
	<i>Citrobacter freundii</i>	Clinical	Portugal	(Ferreira et al., 2010)
	<i>Klebsiella pneumoniae</i>	Clinical	Argentina	(Quiroga et al., 2007)
	<i>Enterobacter cloacae</i>	Clinical	Argentina	(Quiroga et al., 2007)
	<i>Escherichia coli</i>	Clinical	Argentina	(Quiroga et al., 2007)
ISEcp1	<i>Klebsiella pneumoniae</i>	Clinical	Israel	(Navon-Venezia et al., 2008)
	<i>Escherichia coli</i>	Clinical	Israel	(Navon-Venezia et al., 2008)
	<i>Enterobacter cloacae</i>	Clinical	Israel	(Navon-Venezia et al., 2008)
	<i>Proteus mirabilis</i>	Clinical	Israel	(Navon-Venezia et al., 2008)
	<i>Escherichia coli</i>	Clinical	Tunisia	(Ben Slama et al., 2011)
MTn				
Tn21-like	<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i>	Brown trout	France	(Reith et al., 2008)
	<i>Escherichia coli</i>	Dead chicks and turkey poults	China	(Nogrady et al., 2006)
	<i>Salmonella enterica</i> serovar Brandenburg	Clinical and foodborne	Spain	(Martinez et al., 2007)
	<i>Klebsiella oxytoca</i>	Clinical	Spain	(Tato et al., 2010)
	<i>Enterobacter cloacae</i>	Clinical	Spain	(Tato et al., 2010)
Tn5045	<i>Pseudomonas aeruginosa</i>	Permafrost	Russia	(Petrova et al., 2011)
Tn6001	<i>Pseudomonas aeruginosa</i>	Clinical	Taiwan	(Tseng et al., 2007)
GI				
n.n.	<i>Pseudomonas aeruginosa</i>	Clinical	Australia	(Roy Chowdhury et al., 2009)
n.n.	<i>Shigella flexneri</i>	Primate	Japan	(Rajakumar et al., 1997)

(continue)

Table 1 (continued)

MGE	Species	Source of isolation	Country of isolation	Reference
AbaR	<i>Acinetobacter baumannii</i>	Clinical	Australia	(Post and Hall, 2009)
	<i>Acinetobacter baumannii</i>	Clinical	Belgium, Bulgaria, Czech Republic, Spain, Ireland, Italy, Netherlands, Poland, United Kingdom	(Krizova et al., 2011)
SGI	<i>Salmonella enterica</i> serovar Haifa and Newport	Clinical	France	(Doublet et al., 2009)
	<i>Proteus mirabilis</i>	Clinical	Palestine	(Ahmed et al., 2007a)
Plasmid				
n.i.	<i>Enterococcus faecalis</i>	Clinical	United States of America	(Clark et al., 1999)
n.i.	<i>Salmonella enterica</i> serovar Typhimurium	Foodborne	Portugal	(Antunes et al., 2004)
n.i.	<i>Acinetobacter baumannii</i>	Clinical	Taiwan	(Liu et al., 2006)
n.i.	<i>Aeromonas</i> sp.	Wastewater	Portugal	(Moura et al., 2007)
n.i.	<i>Escherichia coli</i>	Wastewater	Portugal	(Moura et al., 2007)
n.i.	<i>Enterobacter cloacae</i>	Clinical	Spain	(Tato et al., 2010)
n.i.	<i>Klebsiella oxytoca</i>	Clinical	Spain	(Tato et al., 2010)
n.i.	<i>Pseudomonas aeruginosa</i>	Clinical	Spain	(Tato et al., 2010)
IncHI2	<i>Enterobacter cloacae</i>	Clinical	Spain	(Tato et al., 2010)
IncI1	<i>Klebsiella pneumoniae</i>	Clinical	Spain	(Tato et al., 2010)
	<i>Escherichia coli</i>	Clinical	Spain	(Tato et al., 2010)

^a GenBank accession number

n.n. – without attributed name; n.i. – without Inc group determined

1.1 Genetic linkage to miniature inverted repeat transposable elements

To our awareness, so far there are only two reports of the presence of MITE-like structures flanking integrons.

Two copies of a 439 bp MITE-like structure have been found flanking the integron of a prawn-associated *Acinetobacter johnsonii*. This integron contains two gene cassettes, one of which is very unusual – it contains three genes, and two of them do not encode antibiotic resistance genes, but methionine sulfoxide

reductases (involved in the repair of proteins damaged by oxidative stress); it was the first time that such genes were found in class 1 integrons (Gillings et al., 2009a).

In the second study, two copies of a MITE-like structure, a 288 bp IMU (integron mobilization unit), were found flanking a defective integron, carrying one antibiotic resistance cassette, in the plasmid of a clinical *Enterobacter cloacae* (Poirel et al., 2009).

1.2 Genetic linkage to insertion sequences

Only a few reports of integrons flanked on each side by an IS can be found in the literature, and these include mainly elements of the IS6 family. For instance: the In53 present in the pNLT-1 plasmid of *E. coli* MG-1 is flanked on each side by IS26 (Naas et al., 2001); the In-t3 present in the pSEM plasmid of a *S. enterica* serovar Typhimurium (Miriagou et al., 2005), the In-111 carried by the pAK33 plasmid of a *Klebsiella pneumoniae* (Miriagou et al., 2005), and one of the integrons of the *S. enterica* serovar Newport 04-3489 (Doublet et al., 2009), are flanked by one IS26 and one IS6100; the integron carried by the p1658/97 plasmid of *E. coli* 1658/97 that is flanked by one complete and one incomplete IS26, followed by another intact IS26 (Zienkiewicz et al., 2007); and the In-e541 present in the p541 plasmid of an *E. coli*, which is flanked by one complete and one incomplete IS26, followed by another intact IS26 that interrupted the 3'-CS of the integron (Miriagou et al., 2005). In some cases, integrons flanked by ISs are incorporated into even larger composite transposons (Miriagou et al., 2005; Doublet et al., 2009).

ISCR1 is always found in complex class 1 integrons (Toleman et al., 2006b, 2006a), and some of the hosts of this element include clinical isolates of *Citrobacter freundii* (Ferreira et al., 2010), *E. cloacae* (Quiroga et al., 2007), *E. coli* (Bae et al., 2007; Quiroga et al., 2007; Song et al., 2010) and *K. pneumoniae* (Quiroga et al., 2007), and *Aeromonas punctata* collected from wastewater (Xia et al., 2010). Another IS conferring 'one-ended transposition' is ISEcp1, which has been associated mainly with the dissemination of the CTX

genes linked with class 1 integrons. For instance, an *ISEcp1* was found upstream of a CTX-M-25 type gene integrated in a class 1 integron in ten Enterobacteria clinical isolates (Navon-Venezia et al., 2008). In another study, the element was associated with a CTX-M-14a-carrying class 1 integron in a clinical *E. coli* strain (Ben Slama et al., 2011). Rolling circle replication is able to mobilise extremely large sections of DNA (Toleman and Walsh, 2011).

1.3 Genetic linkage to transposons

With the exception of the Tn402 (Shapiro and Sporn, 1977; Radstrom et al., 1994) and a few Tn402-like transposons (Labbate et al., 2008; Sajjad et al., 2011), most of the class 1 integrons are considered defective transposons, due to deletions present in DNA regions involved in transposition (Brown et al., 1996; Gillings et al., 2008). However, many integrons are present in functional and mobilisable Tns. For instance, integron In2 (carrying one antibiotic gene cassette) is incorporated in Tn21 (Liebert et al., 1999); many different bacterial species carry Tn21-like transposons with different class 1 integrons, such as isolates of clinical *E. cloacae* and *Klebsiella oxytoca* (Tato et al., 2010), clinical and foodborne *S. enterica* serovar Brandenburg (Martinez et al., 2007), animal pathogenic *E. coli* (Nogrady et al., 2006), and the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* (Reith et al., 2008). There are also many reports of other MTns carrying class 1 integrons; such as the Tn5045 from a permafrost isolate of *P. aeruginosa* (Petrova et al., 2011), the Tn6001 isolated from a clinical *P. aeruginosa* isolate (Tseng et al., 2007), and the Tn5086 from an *E. coli* isolate (Sundstrom et al., 1993).

To date, there are no reports of class 1 integrons carried in ICEs.

1.4 Genetic linkage to genomic islands

In the nosocomial pathogen *A. baumannii*, several resistance islands have been described (AbaR islands), and class 1 integrons are present in many of these.

For instance, the AbaR1 island, described in *A. baumannii* AYE, carries two classic and one complex class 1 integron, with altogether nine resistance gene cassettes (Fournier et al., 2006); the AbaR5, AbaR6 and AbaR7 islands, reported in *A. baumannii* 3208, *A. baumannii* D2 and *A. baumannii* A92, respectively, carry one class 1 integron, coding two antimicrobial resistance genes cassette and two or three additional cassettes with unknown function (Post and Hall, 2009; Post et al., 2010); the AbaR14 island from *A. baumannii* LUH 5881 carries one class 1 integron with one resistance gene cassette (Krizova et al., 2011). Resistance islands are also present in others species, such as the SGI1 island of *S. enterica* serovar Typhimurium DT104 (Hall, 2010) and *Proteus mirabilis* 18306 (Ahmed et al., 2007a), which carry two variants of a complex class 1 integron carrying five resistance genes; the resistance island of *Shigella flexneri* 2a strain YSH6000, containing an incomplete class 1 integron with two gene cassettes (Rajakumar et al., 1997); and the genomic island present in a clinical *P. aeruginosa* strain, which contain transposon Tn6060, formed by two class 1 integrons, carrying a total of six resistance gene cassettes (Roy Chowdhury et al., 2009).

1.5 Genetic linkage to plasmids

There are a high number of reports of class 1 integrons carried by plasmids. For instance, class 1 integrons have been reported residing on plasmids found in the isolates of the Gram-positives *Corynebacterium glutamicum* (Nesvera et al., 1998) and *Enterococcus faecalis* (Clark et al., 1999), and the Gram-negatives *A. baumannii* (Liu et al., 2006), *Aeromonas* sp. (Moura et al., 2007), *E. cloacae* (Tato et al., 2010), *E. coli* (Kang et al., 2005; Moura et al., 2007; Tato et al., 2010), *K. oxytoca* (Tato et al., 2010), *K. pneumoniae* (Tato et al., 2010), *P. aeruginosa* (Tato et al., 2010), and *S. enterica* serovar Typhimurium (Antunes et al., 2004), among other. These reports do not usually indicate the Inc group of the plasmid, which have important implications on the successful HGT of these elements, as plasmids that belong to the same Inc group cannot co-exist in the same cell (Skippington and Ragan, 2011). One of the few studies that

reported the Inc group of the integron-carrying plasmids was done by Tato and collaborators; plasmids belonging to the Inc groups H12 and I1 were shown to carry class 1 integrons (Tato et al., 2010).

A possible limitation in the interpretation of the majority of the studies is that the description of the genomic location of class 1 integrons is usually limited to the plasmid; without further information being provided on the immediate flanking regions of the resistance determinants, such as flanking ISs or MTns. These will likely also be a determinant for the mobility and exact genetic location of class 1 integrons.

2. Evidence of mobility of class 1 integrons

There are many studies that suggest horizontal dissemination of integrons, as the same integron structure is found in different species, in different geographical places and isolated in different periods of time. For example, integron In76, carrying a *bla*_{IMP-5} gene cassette, was isolated in *A. baumannii* 65FFC from Coimbra University Hospital in 1998 (Da Silva et al., 2002), and in eight *P. aeruginosa* isolates collected between 2001 and 2003 from São Bernardo Hospital, Setúbal, and Santa Maria Hospital and Dona Estefânia Hospital, both from Lisbon (Brizio et al., 2006); these isolates were not epidemiologically related (Da Silva et al., 2002; Brizio et al., 2006). Similar is the case of t epidemiologically unrelated *A. baumannii* isolates collected between 1989 and 2000 in six different Italian hospitals that carried an integron with the cassette array *aacC1-orfX-orfX'-aadA1a* (Gombac et al., 2002). The same integron was described in an *A. baumannii* collected in Spain in 2000 (Ribera et al., 2004). Another example includes the presence of the same cassette array integron *dfrA1-aadA1* in the Gram-negatives *E. coli* and *Aeromonas* sp. (Moura et al., 2007), and in the Gram-positives *Corynebacterium ammoniagenes* and *Corynebacterium casei* (Nandi et al., 2004).

3. HGT of class 1 integrons

Some studies directly demonstrate HGT of the class 1 integron-associated MGE, whereas others infer horizontal acquisition of class 1 integron-containing elements, usually by transposition, due to the presence of TSDs.

3.1 HGT due to the linkage with MGEs

3.1.1 Miniature inverted repeat transposable elements

The presence of a 5 bp TSD adjacent to the MITEs-flanking the class 1 integron in *A. johnsonii* NFM2 suggests that the entire structure was acquired through transposition promoted by the MITEs (Gillings et al., 2009a). Experimental mobilization of the entire MITE-integron-MITE structure by transposition was not experimentally observed in this study, which suggests that the transposase required for the movement is not present in that strain. Nonetheless, excision of the integron via homologous recombination between the two MITE copies was observed.

In the study where a defective class 1 integron was surrounded by two IMU in a *E. cloacae* strain, TSD was not present, but the entire structure (IMU-integron-IMU) was shown to transpose and created TSD when a transposase was experimentally provided *in trans* (Poirel et al., 2009).

3.1.2 Insertion sequences

The IS26-composite transposon, named Tn2000, carrying the class 1 integron In53, which integrates an unusual number of gene cassettes (nine), is flanked by TSD, suggesting acquisition of this element by transposition (Naas et al., 2001).

The class 1 integron flanked by one complete and one incomplete IS26, followed by another intact IS26 present in an *E. coli* strain is inserted in a 48 kbs region flanked by different IS26 and similar TSDs, suggesting the acquisition of the entire region by transposition (Zienkiewicz et al., 2007).

The class 1 integron of a *S. enterica* serovar Newport isolate flanked by one IS26 and one IS6100 is inserted in a larger IS26-composite transposon that was inserted into the genomic island SGI1 backbone by transposition, as suggested by the presence of TSDs (Doublet et al., 2009).

3.1.3 Transposons

Transposition of Tn402, a class 1 integron that is also an active transposon, has been observed (Shapiro and Sporn, 1977; Kamali-Moghaddam and Sundstrom, 2000). Another class 1 integron containing a complete transposition module, named Tn6007, was detected in a human commensal strain of *E. cloacae*; Tn6007 with a single base pair mutation in the *tniA* gene, present in a fosmid clone, was shown to transpose in the presence of another *tniA* gene *in trans* – TSDs flanked both ends of the Tn6007 (Labbate et al., 2008).

Transposition of Tn21 and Tn21-like transposons has been widely observed, which contributes to the broad dissemination of class 1 integrons (de la Cruz and Grinsted, 1982; Grinsted et al., 1990).

The TSD flanking the class 1 integron InC* and the TSD flanking the integron-carrying transposon Tn5045 suggest two transposition events in the generation and acquisition of this transposon by the chromosome of the permafrost isolate *Pseudomonas* sp. Tik3 (Petrova et al., 2011).

Another example of mobility of class 1 integrons due to the linkage with a MTn includes the experimentally observed transposition of a class 1 integron-carrying transposon, Tn5086, carried by an *E. coli* isolate (Sundstrom et al., 1993).

3.1.4 Genomic islands

A recent study demonstrated the mobilization *in trans* of the SGI1 island, containing the integron In104, from two strains of *S. enterica* serovar Agona to *E. coli* by conjugation; only plasmids from the IncA/C incompatibility group were able to mobilise this GI (Douard et al., 2010).

The disruption of the gene *comM* and the presence of a 5 bp TSD flanking the entire GI, are strong indicators that the class 1 integron-carrying AbaR1, AbaR3 and AbaR5 islands, present in different strains of *A. baumannii*, have been acquired by transposition (Post et al., 2010).

3.1.5 Plasmids and conjugation

Several studies have reported on the transfer of class 1 integrons by conjugation due to the linkage with plasmids; studies were done in several different species and genera.

For instance, Meng and colleagues (Meng et al., 2011) demonstrated a plasmid-mediated transfer of two class 1 integrons, using *S. enterica* serovar Derby HBS010 and *S. enterica* serovar Indiana HBS084, isolated from pork and chicken food, respectively, as donors and *E. coli* RG488 as recipient; the conjugative plasmid transfer occurred at a frequency of 10^{-5} - 10^{-6} .

The reference strain *Yersinia enterocolitica* CECT4054 from a Spanish collection was shown to carry a class 1 integron in a self-transmissible plasmid, pUO-Ye-R1, with 140 kb; transfer of the plasmid to the recipient *E. coli* K12 J53 by conjugation was demonstrated (Soto et al., 2003).

Clinical isolates of *Serratia marcescens* were shown to harbour class 1 integron-carrying plasmids; three different plasmids, and respective class 1 integron, were transferred to *E. coli* HB101 by conjugation (Hu and Zhao, 2009). Also Moubareck and collaborators (Moubareck et al., 2009) showed transfer of the integron-carrying plasmid pIP847 (90 kbs) from *A. baumannii* BM4674 to *A. baumannii* BM4652 by conjugation.

Tato and colleagues (Tato et al., 2010) observed that plasmids belonging to the IncHI2 and IncI1 groups were detected in transconjugants after conjugation with clinical isolates of *E. cloacae* and *E. coli* as donors, showing the capacity of these plasmids to horizontally move and disseminate class 1 integrons; transfer of integron-carrying plasmids from other species were also detected in this study, but the Inc group of the plasmids was not determined.

The gene cassettes transferred in the plasmid-borne integrons, described in the previous studies, were expressed in the transconjugants.

3.1.6 Bacteriophages and transduction

As bacteriophages can transfer random fragments of host DNA to the infected bacterial cell during transduction, it is expected that also DNA fragments with integrons can be horizontally transferred to other cells by this mechanism. To our awareness, there is only one report on the role of transduction as a mechanism of horizontal transfer of resistance genes embedded in integrons, in *S. enterica* serovar Typhimurium DT104. In the study, an *amp*-containing integron was shown to be co-transduced when selection for other resistance genes was done; generalized transduction promoted by a temperate phage was the HGT mechanism involved (Schmieger and Schicklmaier, 1999).

It should, however, be noted that retrospective studies might not be able to identify transduction as a causal mechanism for DNA transfer, as there is no specific genetic signature for events of DNA acquisition by transduction.

3.1.7 Natural transformation

As any fragment of DNA can be taken up over the bacterial membrane of most of the competent cells, natural transformation could contribute to acquisition of class 1 integrons.

The uptake of self-replicating plasmids by natural transformation has the potential to disseminate linked integrons; only few studies were dedicated to the

plasmid-mediated transfer of integrons via natural transformation. For example, Meng and collaborators (Meng et al., 2011) demonstrated the transfer of two integron-carrying plasmids, one from *S. enterica* serovar Derby HBS010 and one from *S. enterica* serovar Indiana HBS084, isolated from pork and chicken food, respectively, to the oral cariogenic naturally competent *Streptococcus mutans* UA159, via natural transformation; the transformation frequency was in the order of 10^{-6} - 10^{-7} .

A complex class 1 integron located in the pDCMSR1 plasmid of the clinical *P. mirabilis* Prm9 was also shown to transform the clinical natural competent *A. baumannii* strain A118 and to be stably maintained in the recipient cell for at least 40 generations (Ramirez et al., 2012).

Less is known about the transformation potential of integrons present in plasmids incompatible with the new host or when present in chromosomal locations.

A recent study with synthetic gene cassettes demonstrated acquisition of gene cassettes by natural transformation. The two natural competent strains of *P. stutzeri*, 17587 and 17641, contained an integron located in the chromosome; both strains were transformed with different types of circular cassettes and linear gene cassette arrays. The linear gene cassette array transformed *P. stutzeri* at a higher frequency than circular cassettes; the vast majority of the cassettes were integrated at the *attI* site. Cassettes without the *attC* site did not transform the recipient cells successfully, which indicates that cassettes were acquired by site-specific recombination, and not by homologous or illegitimate recombination (Gestal et al., 2011).

3.2 Mobility of class 1 integrons independent of genetic linkage to MGEs

3.2.1 Movement of class 1 integrons supported by elements *in trans*

Some authors suggest that class 1 integrons can move independently under appropriate circumstances, such as in the presence of a complete transposition (*tni*) module provided *in trans* and in the presence of the two inverted repeats IR_i and IR_t flanking the class 1 integron (Brown et al., 1996; Partridge et al., 2001b). However, only few studies have reported experimental evidence of horizontal transfer of class 1 integrons without the genetic linkage to other MGEs.

Mobility of integrons by transposition-like mechanisms has been shown for a few integrons with transposition related genes. Transposon Tn2521, identified in clinical strains of *P. aeruginosa*, has been shown to have the ability to transpose (Sinclair and Holloway, 1982); this transposon was later described to be a class 1 integron, renamed In33, and lacking transposition genes (Partridge et al., 2002). Further analysis of the recipient revealed the presence of a 5 bp direct TSD, which confirms the transfer of In33 by transposition. It was suggested that the transposition event was supported by *tni* genes from another transposon present in the same cell, although this was not experimentally shown (Partridge et al., 2002).

In another study, experimental transposition of class 1 integrons lacking a transposition module, In0 and In2, was observed when assisted by *tni* genes from transposon Tn502 provided *in trans*; transposition was confirmed by the presence of a 5 bp direct TSD flanking the integrons in their new location (Petrovski and Stanisich, 2010).

3.2.2 Movement of class 1 integrons and gene cassettes by homologous recombination

As the conserved regions of class 1 integrons provide sufficient stretches of DNA similarity for recombinational exchange between two integron-containing bacteria, homologous recombination has been suggested to contribute to the replacement of the gene cassette arrays in class 1 integrons (Hall and Collis, 1995). For example, such recombination could explain the loss of the gene cassette array of *In3* when *Tn1405* was acquired by the *In3*-containing R388 plasmid (Partridge et al., 2002). Integration of DNA by homologous recombination can occur after the three HGT mechanisms (Skippington and Ragan, 2011); however, the role of homologous recombination in the replacement of gene cassettes has not been experimentally shown.

VII. Limitations to the dissemination of class 1 integrons

Genetic linkage to MGEs enables class 1 integrons to transfer horizontally within and between bacterial populations and species. The limiting factors to dissemination of integrons will therefore in most cases be similar to those governing the dissemination of the MGE they are linked to. Typically, transfer by conjugation will be limited by mechanistic aspects of the conjugal machinery as well as compatibility of the plasmid with the new host and its restriction-modification system (Mazodier and Davies, 1991; Thomas and Nielsen, 2005). For transduction, key limiting factors are the host range of the transducing bacteriophages as well as the host restriction-modification system (Mazodier and Davies, 1991). For natural transformation, key limiting factors are the ability to develop competence for DNA uptake as well as the ability of incoming DNA to recombine with the genome of the transformed cell (Thomas and Nielsen, 2005). In order to be vertically inherited, non-replicative DNA with class 1

integrons transferred, taken up or injected into the bacterial cytoplasm must be able to recombine with the host genome. Such recombinational barriers are the same as those preventing HGT of any chromosomal DNA between the genomes of non-related bacteria (Frost et al., 2005; Thomas and Nielsen, 2005).

A general limiting factor, beyond the mechanistic aspects defining the probability of transfer to new hosts, is the likelihood that the acquired MGE will become vertically inherited in the larger bacterial population. It is known that initially rare events of HGT are unlikely to be established in a larger bacterial population if they do not confer a benefit to the host (Pettersen et al., 2005; Townsend et al., 2012). Thus, in cases where the acquisition of integron-carrying MGE will negatively affect the relative or absolute growth rate of the new host, rare HGT events will be lost from the larger population as they will be outgrown by more fit members of the population. At high transfer rates, a temporal equilibrium of integron-carrying MGEs in the population can be established when the frequency of new successful HGT events equals the loss of non-fit members (Harrison and Brockhurst, 2012). However, several studies have shown that both the MGEs and the bacterial host can rapidly co-adapt; so that the initial high fitness costs to the host can, within weeks, be reduced or lost. Eventually, the MGE-carrying hosts can be equally or more fit than the remaining bacterial population (Dionisio et al., 2005; Johnsen et al., 2011; Harrison and Brockhurst, 2012). This amelioration of the fitness costs is due to compensatory mutations (Bjorkman and Andersson, 2000; Andersson, 2003).

In cases where the class 1 integron-carrying MGE acquisition does not result in an initial fitness change in the new host (Enne et al., 2005), the frequency of successful transfer, and random fluctuations in the bacterial population (genetic drift), will determine the short term-fate of the new class 1 integron-carrying host cells. However, in cases where co-adaptation takes place between the MGE/class 1 integron and the host genome, more complex populations trajectories can be expected (Geissler et al., 2003; Bergmiller et al., 2012). Whereas the bacterial mutation rate is well understood (Denamur and Matic, 2006), less is known about the various mechanisms leading to deletions of

acquired DNA segments in the bacterial genome. In cases where the integron-containing MGE confers a growth advantage to the new host, even very few HGT events can be sufficient for establishing the class 1 integron in the new host (Enne et al., 2005). For instance, the use of antimicrobial drugs in the treatment of bacterial infections can effectively remove drug sensitive cells, so that even exceedingly low initial proportions of integron-containing cells can rapidly expand their population size to become the dominant one. Dominant populations of resistance-carrying bacterial pathogens are a major public health issue.

VIII. Aims of the study

The plasticity and adaptation of the bacterial genome is intrinsically related with MGEs, which have a key role in the spread of antibiotic resistance genes among bacterial populations. Integrons themselves, especially those belonging to class 1, are also suggested to have an important role in the dissemination of antibiotic resistance genes, due to their high prevalence in diverse bacterial populations. Class 1 integrons are usually found linked to various types of MGEs; and will, therefore, move within and between bacterial genomes as part of the MGE they reside in (Levin and Bergstrom, 2000), in a process called hitchhiking. Although integrons embedded in Tns and ISs are considered to have intragenomic mobility only, their further intergenomic dissemination can be facilitated by the horizontal mobility of the genetic element or the genetic region they reside, drawing on both transduction and conjugational processes. Important considerations for understanding the fate of integrons include their genetic context, the dissemination rates of linked MGEs, expression levels of integrons and gene cassettes and absolute and relative host fitness effects.

The horizontal movement of class 1 integrons genetically linked to non-conjugative elements (Liebert et al., 1999) or incomplete mobile genetic

elements and transposons (Brown et al., 1996; Stokes et al., 2006; Tato et al., 2010) remains to be explained. For instance, the conserved regions of class 1 integrons could provide sufficient DNA sequence similarity for homologous recombination to occur. However there are no studies demonstrating the role of homologous recombination in the exchange of gene cassettes between class 1 integron-containing bacteria. Only few studies have attempted to experimentally examine how (non-conjugative) transposons, integrons or gene cassettes move horizontally. These include reports on the recruitment of gene cassettes (Rowe-Magnus et al., 2002), the acquisition and integration of synthetic gene cassettes by natural transformation (Gestal et al., 2011) and transposition of the integron In33 (Sinclair and Holloway, 1982; Partridge et al., 2002).

We aimed to determine the potential for class 1 integrons and gene cassettes to move horizontally between bacterial species via natural transformation, and reveal any genetic linkage between the acquired integrons and MGEs. To experimentally determine the transfer potential, the integron-free and naturally-transformable bacterium *A. baylyi* was exposed to DNA obtained from diverse class 1 integron-carrying Gram-negative bacteria.

The specific objectives of this study were:

- To determine the presence, and to analyse the DNA sequence and resistance pattern, of class 1 integrons in selected bacterial isolates;
- To test the hypothesis that natural transformation is involved in class 1 integron transfer;
- To analyse the genomic context of class 1 integrons in order to identify flanking sequences and the genetic linkage possibly related with their movement;
- To assess the *in vitro* fitness cost conferred by interspecies transfer of class 1 integrons by natural transformation;

- To identify other possible genes (whole or fragments) that can be transferred by natural transformation and affect the antimicrobial susceptibility profile of the bacteria.

Materials and Methods

I. Bacterial strains

Wild-type bacteria, transformants and constructed strains were used in this study. Except when specified, the bacteria were grown in Luria-Bertani (LB) medium, supplemented with the appropriate antibiotics (specified for each isolate in the follow subsections). *A. baylyi* cells were grown at 30 °C, and the different wild-type bacteria at 37 °C.

1. Donor bacteria

The wild-type class 1 integron-carrying bacteria with diverse antibiotic resistance gene cassettes (indicated in parenthesis) used as DNA donors were the following: *A. baumannii* 064 (*aadB*), *A. baumannii* 65FFC (*bla*_{IMP-5}) and *P. aeruginosa* SM (*aacA4-bla*_{PSE}-*aadA2*) (*P. aeruginosa* SM was a kind gift from Prof. Dr. Sónia Mendo), all clinical isolates; *S. enterica* serovar Rissen 486 (*dfrA12-aadA2*) and *S. enterica* serovar Typhimurium 490 (*bla*_{OXA-30}-*aadA1*), both isolated from pork processed food; and *C. freundii* C16R385 (*dfrA12-orfF-aadA2*), *E. cloacae* C2R371 (*dfrA12-orfF-aadA2*), *E. coli* C10R379 (*dfrA7*), and *Escherichia fergusonii* AS041A2 (*dfrA1-aadA1*) isolated from food-producing and wild animals (a kind git from Prof. Dr. Maria José Saavedra).

The multiresistant clinical isolates *E. coli* K71-77, *K. pneumoniae* K66-45 and *P. aeruginosa* K34-73 (a kind gift from Dr. Ørjan Samuelsen) were also used as donor DNA, but only to evaluate the phenotypic changes of the transformants.

Wild-type donor bacteria were grown in LB supplemented with antibiotics according to their resistance phenotype: *A. baumannii* 064 in kanamycin (K) 50 µg/ml; *A. baumannii* 65FFC in ampicillin (AM) 50 µg/ml or cefotaxime (CTX) 50 µg/ml; *P. aeruginosa* SM in AM 50 µg/ml or spectinomycin (SC) 50 µg/ml; *S. enterica* serovar Rissen 486 in SC 50 µg/ml; *S. enterica* serovar Typhimurium 490 in SC 50 µg/ml; *C. freundii* C16R385 in SC 50 µg/ml; *E. cloacae* C2R371 in SC 50 µg/ml; *E. coli* C10R379 in trimethoprim (W) 50 µg/ml; *E. fergusonii*

AS041A2 in W 50 µg/ml; *E. coli* K71-77 in gentamicin (CN) 50 µg/ml; *K. pneumoniae* K66-45 in CN 50 µg/ml; and *P. aeruginosa* K34-73 in CN 50 µg/ml. Six transformants of *A. baylyi*, isolates SD1 (*bla*_{OXA-30}-*aadA1*), SD2 (*aadB*), SD3 (*bla*_{IMP-5}), SD4 (*bla*_{OXA-30}-*aadA1*), SD5 (*dfrA12-aadA2*) and SD6 (*aacA4-bla*_{PSE}-*aadA2*), obtained by natural transformation in this work, and that were confirmed to have acquired a class 1 integron from a heterologous donor source, were also used as donor DNA in subsequent transformation assays. In addition, the *A. baylyi* transformant KOI(Ps)1, an isolate containing an integron obtained by natural transformation of KOI with DNA from *P. aeruginosa* SM, and containing an inactive integrase, was used as a source for donor DNA. Transformants were grown in LB supplemented with rifampicin (R) 25 µg/ml plus: SC 10 µg/ml for SD1, SD4, SD5, SD6 and KOI(Ps)1; K 10 µg/ml for SD2; and AM (25 µg/ml) or CTX 10 µg/ml for SD3. All strains used as DNA donors in this study and main traits are summarized in Table 2.

Table 2. Donor DNA strains used in natural transformation in this study.

Strain	Origin	Plasmid	Integron size (bp) ^a	Gene cassettes	Relevant phenotype/trait ^b	Reference
<i>A. baumannii</i> 064	Clinical	Yes	762	<i>aadB</i>	K ^R	(Domingues, 2008)
<i>A. baumannii</i> 65FFC	Clinical	No	1000	<i>bla</i> _{IMP-5}	CTX ^R	(Da Silva et al., 2002)
<i>A. baylyi</i> SD1	Transformant	No	2000	<i>bla</i> _{OXA-30} - <i>aadA1</i>	SC ^R	This study
<i>A. baylyi</i> SD2	Transformant	No	762	<i>aadB</i>	K ^R	This study
<i>A. baylyi</i> SD3	Transformant	No	1000	<i>bla</i> _{IMP-5}	CTX ^R	This study
<i>A. baylyi</i> SD4	Transformant	No	2000	<i>bla</i> _{OXA-30} - <i>aadA1</i>	SC ^R	This study
<i>A. baylyi</i> SD5	Transformant	No	1912	<i>dfrA12</i> - <i>aadA2</i>	SC ^R	This study
<i>A. baylyi</i> SD6	Transformant	No	3000	<i>aacA4</i> - <i>bla</i> _{PSE} - <i>aadA2</i>	SC ^R	This study
<i>A. baylyi</i> [KOI](Ps)1	Transformant	No	3000	<i>aacA4</i> - <i>bla</i> _{PSE} - <i>aadA2</i>	SC ^R ; Δ <i>int1</i>	This study
<i>C. freundii</i> C16R385	Rabbit	Yes	1913	<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>	SC ^R	This study
<i>E. cloacae</i> C2R371	Rabbit	Yes	1913	<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>	SC ^R	This study
<i>E. coli</i> C10R379	Rabbit	No	769	<i>dfrA7</i>	W ^R	This study
<i>E. coli</i> K71-77 ^c	Clinical	Yes	n.d.	-----	CN ^R	(Samuelsen et al., 2011)
<i>E. fergusonii</i> AS041A2	Owl	Yes	1594	<i>dfrA1</i> - <i>aadA1</i>	W ^R	This study
<i>K. pneumoniae</i> K66-45 ^c	Clinical	Yes	n.d.	-----	CN ^R	(Samuelsen et al., 2011)
<i>P. aeruginosa</i> K34-73 ^c	Clinical	No	3604	<i>bla</i> _{VIM-4} - <i>arr7</i> - <i>aacA4</i> - <i>aadA1</i>	CN ^R	(Samuelsen et al., 2010)
<i>P. aeruginosa</i> SM	Clinical	No	3000	<i>aacA4</i> - <i>bla</i> _{PSE} - <i>aadA2</i>	SC ^R	(Caetano et al., 2007)
<i>S. enterica</i> Rissen 486	Fresh pork sausage	Yes	1912	<i>dfrA12</i> - <i>aadA2</i>	SC ^R	(Domingues, 2008)
<i>S. enterica</i> Typhimurium 490	Pork hamburger	Yes	2000	<i>bla</i> _{OXA-30} - <i>aadA1</i>	SC ^R	Da Silva, unpublished

^a Obtained with 5'-CS and 3'-CS primers (Levesque et al., 1995).

^b The resistance trait was used for selection in the natural transformation assays.

^c Transformants obtained after exposure to these sources of DNA were only tested at the phenotypic level.

^R Resistance; CN – gentamicin, CTX – cefotaxime, K – kanamycin, SC – spectinomycin, W – trimethoprim.

n.d. – not determined.

2. Recipient bacteria

The naturally competent soil bacterium *A. baylyi* BD413 (Juni, 1972), rifampicin resistant (R^R) (Nielsen et al., 1997), was used as the recipient cell in natural transformation assays. The genome of the closely-related *A. baylyi* ADP1 has been sequenced (accession number CR543861) and published (Barbe et al., 2004). The properties of this strain, including natural competence for transformation, the absence of specific sequence requirements for DNA uptake,

the small genome (3.6 Mb) and the rapid growth in laboratory make it an excellent laboratory model that is easy to genetically modify (Palmen et al., 1993; Barbe et al., 2004). *A. baylyi* BD413 was cultivated in LB with R 50 µg/ml. Close derivatives of *A. baylyi* BD413, obtained during this work, were also used as recipient cells: the integron-carrying *A. baylyi* transformant SD2; the integron-carrying with partial integrase deletion *A. baylyi* KOI ($\Delta int1$); the integron-carrying *A. baylyi* *recA* deletion RAM ($\Delta recA$); the double mutant strain *A. baylyi* SD9 ($\Delta recBCD \Delta sbcCD$); the competence mutant strain *A. baylyi* KOC4 ($\Delta comFECB$); and the *tnp* (*tnpA*, *tnpR* and *tnpM*) module-carrying construct *A. baylyi* SD12 (ACIAD3309::*tnp* module).

The *A. baylyi* transformant SD2 was obtained by natural transformation of *A. baylyi* BD413 with *A. baumannii* 064, and carried a class 1 integron with the gene cassette *aadB*. The *A. baylyi* KOI, the *A. baylyi* RAM, and the *A. baylyi* SD12 were constructed using cloning and natural transformation strategies; the double mutant *A. baylyi* SD9 and the non-competent *A. baylyi* KOC4 were constructed by natural transformation; see below for details.

All strains used as recipient cells in this study and main traits are summarized in Table 3.

Table 3. Recipient strains used in natural transformation in this study.

Strain	Origin	Plasmid	Integron size ^a (bp)	Gene cassettes	Relevant phenotype / trait ^b	Reference
<u>Recipients:</u>						
<i>A. baylyi</i> BD413	Soil	No	No	-----	R ^R	(Juni, 1972; Nielsen et al., 1997)
<i>A. baylyi</i> SD2	Transformant	No	762	<i>aadB</i>	K ^R	This study
<i>A. baylyi</i> KOI	Transformant	No	762	<i>aadB</i>	K ^R ; $\Delta int1$	This study
<i>A. baylyi</i> RAM	Transformant	No	762	<i>aadB</i>	K ^R ; $\Delta recA$	This study
<i>A. baylyi</i> SD9	Transformant	No	No	-----	K ^R ; $\Delta recBCD \Delta sbcCD$	This study
<i>A. baylyi</i> KOC4	Transformant	No	No	-----	W ^R ; $\Delta comFECB$	This study
<i>A. baylyi</i> SD12	Transformant	No	No	-----	Suc ^R ; <i>tnpA+R+M</i>	This study

^a Obtained with 5'-CS and 3'-CS primers (Levesque et al., 1995).

^b The resistance trait was used for selection in the natural transformation assays.

^R Resistance; K – kanamycin, R – rifampicin, Suc – sucrose, W – trimethoprim.

n.d. – not determined.

2.1 Construction of recipients

Construction of recipients was done by cloning of DNA in *E. coli* cells followed by natural transformation of *A. baylyi* BD413 or SD2, or only by natural transformation of *A. baylyi* BD413.

Different antibiotics, and other compounds, were used to select and grow the transformed cells (specified in the description of the construction of each recipient).

Vectors used in the construction of the recipients (details below) were digested with 10 U of restriction enzymes *Ecl136II*, *HincII* or *ScaI* from Fermentas or *KpnI*, *SacI*, *XhoI* or *XmnI* from New England Biolabs; incubation temperatures and inactivation of the enzymes were used according to the manufacturer's instructions. Whenever heat inactivation was not possible, the linearized vector was recovered by gel extraction. Incubation times varied from 1 hr to overnight (ON) digestion.

PCRs performed to check insertion and orientation of the segments incorporated in transformed cells, as specified below, were done with a *Taq* DNA polymerase with the reaction mix described in the section V, subsection 1. The primers used are listed on Table 4.

2.1.1 Methods used in the construction of recipient strains

Production of DNA constructs for insertion into *A. baylyi* was done by cloning in *E. coli*. PCR-products used in the cloning procedures were amplified with the high-fidelity DNA polymerase Phusion, as explained in section V, subsection 1, with the primers listed on Table 4, and purified as explained in the appropriate section (V, subsection 3).

Ligation of the vector and the insert was done with T4 DNA ligase (Fermentas or New England Biolabs), in a ligation mix with total volume of 10 μ l, containing 1 μ l of buffer 10 x, 4 U of ligase, and vector and insert in a 1:1 ratio. Ligation occurred during 1 hr or ON at room temperature; inactivation of the ligase was done at 65 °C for 10 min.

Introduction of DNA into *E. coli* was done either by chemical transformation (*E. coli* cells involved in the construction of *A. baylyi* SD12) or by electroporation (*E. coli* cells involved in the construction of *A. baylyi* RAM).

Introduction of DNA into *A. baylyi* was done by natural transformation in liquid culture (liquid transformation) or in nitrocellulose membrane filters (filter transformation, described in section III, subsection 1). Filter transformation was used to construct the isolates *A. baylyi* KOI and RAM and liquid transformation was employed in the construction of isolates *A. baylyi* SD9, SD11, SD12 and KOC4.

a) Chemical transformation

Chemical transformation of *E. coli* was done based on the protocol described by Inoue and collaborators (Inoue et al., 1990). *E. coli* EC100 and DH5 α were the artificially competent strains used.

E. coli strains were grown in 250 ml of SOB medium (2 % Bacto-tryptone, 0.5 % Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄) at 18 °C with good aeration, until the OD (optical density) at 600 nm reached 0.6. After 10 min on ice, cells were centrifuged at 4000 rpm in a SS-34 rotor (Sorvall) for 10 min at 4 °C. The pellet was then resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂, 250 mM KCl) and kept on ice for 10 min. A new centrifugation was done (same conditions) and the pellet was resuspended in 20 ml of TB. DMSO was added to obtain a final concentration of 7 % and the suspension was kept on ice for 10 min. Competent cells were frozen in 650 μ l aliquots in liquid nitrogen, and stored at - 80 °C until used.

The competent cells were thawed at room temperature and aliquots of 200 μ l were dispensed in 1.5 ml Eppendorf tubes. Plasmid or ligation mix was added (maximum 5 μ l) and maintained on ice for 30 min, and afterwards heated at 42 °C for 1 min and transferred again to ice. A volume of 800 μ l of SOC (1/100 volume [vol] of 2 M glucose added to SOB) was added to the cells and incubated at 37 °C for 1 hr, with shaking. Finally, the transformation suspension

was plated on LB agar with appropriate selective antibiotic and incubated ON at 37 °C.

b) Electroporation

The electroporation protocol was modified from Dower and collaborators (Dower et al., 1988). *E. coli* EC100 and DH5 α were the artificially competent strains used.

The first step was the preparation of the competent cells. An ON culture of *E. coli* was diluted 1:100 in LB and incubated at 37 °C on a shaker until the cell titer reached 2×10^8 cells/ml (calculated with a counting chamber/hemocytometer). The cell culture was then cooled on ice, transferred to pre-cooled centrifuge beakers and centrifuged for 8 min at 5000 \times g at 4 °C. The cells were washed twice with 1 vol of pre-cooled 10 % glycerol and centrifuged again. Cells were resuspended in 0.002 vol of pre-cooled 10 % glycerol, which gives a cell titer of 1×10^{11} cells/ml. Aliquots of 40 μ l were distributed into Eppendorf tubes and immediately frozen at - 80°C.

A volume of 40 μ l of competent cells was used in each electroporation assay. After thawed on ice, 1 μ l (or 0.5 μ l if the salt content was too high) of DNA or ligation mix was added and incubated on ice for approx. 1 min. The electroporator (Gene Pulser Xcell, BioRad) was set with appropriate parameters for a 2 mm-cuvette (voltage 2.5 kV, resistance 200 Ω ; capacity 25 μ F). The cell-DNA mix was transferred into the pre-cooled electroporation cuvette, quickly put into the slot of the electroporator, and pulsed immediately. Cells were recovered from the cuvette with 1 ml of LB and placed in a glass tube, which was further aerated for 1 hr at 37 °C. Finally the suspension was plated on selective LB agar medium and incubated ON at 37 °C.

c) Liquid natural transformation

Liquid transformation was performed based on the protocol published by Harms *et al.* (Harms *et al.*, 2007).

A 5 ml culture of the recipient was prepared from one single colony, and incubated at 30 °C with agitation at 150 rpm ON. The following day, an 1:100 dilution was done in a final volume of 5 ml and aerated (150 rpm) at 30 °C to 1×10^9 cells/ml, which took approx. 6 hrs. Cells were pelleted down by centrifugation in a SS-34 rotor (Sorvall) for 15 min at 4000 rpm, 4 °C, and resuspended in 4 vol of fresh LB; each ml contains 2.5×10^8 cells. Aliquots with 1 ml were transferred to glass tubes and 1 µg of DNA was added, the solution aerated (150 rpm) at 30 °C for 90 min, and plated in LB with appropriate selection. Plates were incubated at 30 °C. Transformed cells were checked 1 or 2 days later.

2.1.2 Detailed construction of the recipient strains

A. baylyi KOI

To construct the *A. baylyi* KOI ($\Delta int11$), the pACYC177-*int-cat* (containing the *int11* gene interrupted by the chloramphenicol resistance gene *cat*) constructed by Starikova *et al.* (Starikova *et al.*, 2012) was *HincII*-linearized and used to naturally transform *A. baylyi* SD2, giving the strain KOI; transformed cells were selected in LB agar with K 10 µg/ml and chloramphenicol (Cl) 10 µg/ml.

The disruption of the *int11* gene in the isolate KOI was verified by PCR with three combinations of primers, binding in the *cat*, *aadB*, *int11* and *tnpA* (adjacent to the *int11* gene) genes: HS464 + AADB2, HS463a + TNPAF1, and AADB2 + *cat-r*. The PCR program included an initial step at 94 °C for 7 min, 30 cycles of 30 sec at 94 °C, 1 min at 58 °C and 3 min at 72 °C, and a final extension step at 72 °C for 10 min. Product amplification with AADB2 + *cat-r* and an amplicon with HS464 + AADB2 and HS463a + TNPAF1 larger than the obtained when

using DNA from the control *A. baylyi* SD2 indicated the acquisition of an isolate with inactivated integrase.

A. baylyi RAM

In the *A. baylyi* RAM ($\Delta recA$) construction, an internal 938 bp segment of the *recA* gene of *A. baylyi* BD413 was PCR-amplified with primers *recA*-f and *recA*-r. The *recA* segment was inserted into the *HincII* site (into the *lacZ'* gene, which gives a blue color to the colonies, in the presence of IPTG and X-gal) of pUC19, giving pUC19-*recA*; and introduced into *E. coli* EC100 by electroporation. Transformed cells were selected in LB agar with AM 100 $\mu\text{g/ml}$, IPTG 40 $\mu\text{g/ml}$ and X-gal 40 $\mu\text{g/ml}$; colonies that acquired the vector pUC19-*recA* showed a white coloration (once the insert interrupts the *lacZ'* gene). Plasmids were isolated from a few white colonies and a restriction analysis with *KpnI* was done, to confirm the acquisition of the vector with the insert, and to check the orientation of the fragment. A segment (1077 bp) containing the *cat* gene from the vector pACYC184 was PCR-amplified using the primers *cat*-f and *cat*-r. The *cat* segment was cloned into the singular *HincII* site of pUC19-*recA*, now located in the center of the *recA* fragment, resulting in pUC19-*recA*-*cat*. This vector was introduced into *E. coli* EC100 by electroporation and transformed cells were selected for in LB with Cl 25 $\mu\text{g/ml}$. Extraction of plasmids was done and a restriction analysis with *Scal* was performed. The pUC19-*recA*-*cat* vector was then *XmnI*-linearized and used to inactivate the *recA* gene of *A. baylyi* SD2 (K^R) by natural transformation, yielding the strain RAM; selection of the transformed cells was done in LB agar with K 10 $\mu\text{g/ml}$ and Cl 10 $\mu\text{g/ml}$.

The correct inactivation of the *recA* gene was verified by PCR with four combinations of primers, binding in the *recA* and *cat* genes: *recA*-f + *cat*-r, *recA*-f + *cat*-f, *recA*-r + *cat*-r, and *recA*-r + *cat*-f. The PCR program included an initial step at 94 °C for 7 min, 30 cycles of 30 sec at 94 °C, 1 min at 56 °C and 2 min at 72 °C, and a final extension step at 72 °C for 10 min. The presence of an amplicon with primers *recA*-f + *cat*-f and with *recA*-r + *cat*-r and the absence with the other two set of primers indicated the successful knock-out of the *recA* gene.

A. baylyi SD9

The double mutant *A. baylyi* SD9 ($\Delta recBCD \Delta sbcCD$) was constructed as described by Harms and Wackernagel (Harms and Wackernagel, 2008). Briefly, plasmids pKH80 [$\Delta sbcCD::(nptII \ sacB)$], pKH81 ($\Delta sbcCD$) and pKH83 [$\Delta recBCD::(nptII \ sacB)$] were digested with *Xho*I and naturally transformed *A. baylyi* cells.

First, *A. baylyi* BD413 was naturally transformed with the linearized pKH80 (500 ng), producing an *A. baylyi* transformed cell K^R (10 μ g/ml) and sucrose sensitive (Suc^S) (50 g/l), SD7. Subsequently, *A. baylyi* SD7 was naturally transformed with the linearized pKH81 (500 ng) to produce the *A. baylyi* SD8, K^S (10 μ g/ml) and Suc^R (50 g/l). Finally, *A. baylyi* SD8 was naturally transformed with the linearized pKH83 (500 ng) to generate the *A. baylyi* SD9, a double mutant K^R (10 μ g/ml) and Suc^S (50 g/l).

The correct inactivation of the *recBCD* genes was tested by PCR with primers Ping0 and *recD-down-r*; the primers *sbcD-f11* and *sbcC-r18* were used to determine inactivation of the *sbcCD* genes. The PCR program included an initial step at 94 °C for 5 min, 30 cycles of 1 min at 94 °C, 1 min at 58 °C and 2 min at 72 °C, and a final extension step at 72 °C for 10 min. PCR-products of 1.3 kb and 0.2 kb were indicative of a deletion in the *recBCD* and *sbcCD* operons in *A. baylyi* SD9, respectively. *A. baylyi* BD413 was used as the control, and no product should be obtained with the first pair of primers, while a 5 kbs product would be obtained with the second one, but with an extension time of 4 min (though a *Taq* DNA polymerase might not be able to amplify such length; in that case, absence of a PCR-product is considered the expected result).

A. baylyi SD11

A. baylyi SD11 was produced by transformation of *A. baylyi* BD413 with 1 μ g DNA from *A. baylyi* IVS4 (*ACIAD3309::nptII sacB*; K^R and Suc^S) (Starikova et al., 2012). Transformed cells were selected in LB with K 10 μ g/ml and checked for Suc^S (50 g/l).

Insertion of the *nptII-sacB* genes in the *ACIAD3309* gene of *A. baylyi* was confirmed by PCR, with the Phusion polymerase, using the primers DownR and

UpF2. The recipient *A. baylyi* BD413 was used as control (PCR-product of 2 kb); PCR-product with approx. 4 kbs indicated the acquisition of the *nptII-sacB* segment by the strain *A. baylyi* SD11.

A. baylyi SD12

The construction of *A. baylyi* SD12 (ACIAD3309::*tnp* module) included the introduction of a *tnp* module in *A. baylyi* SD11. Amplification of the *tnp* module, including the *tnpA*, *tnpR* and *tnpM* genes from *S. enterica* serovar Typhimurium 490, was done with primers TNP1 and TNP2. The PCR-product was then cloned in the *Ecl136II*-site of pTM4 (Starikova et al., 2012) located inside the ACIAD3309 gene (homologous to the ACIAD3309 gene of *A. baylyi* BD413), resulting in pTM4-*tnp*. This vector was digested with *SacI* (same recognition site as *Ecl136II*), to exclude re-ligated vector without insert, once there is no marker in the insert that allowed selection, and the insert-containing vector does not have the *SacI* recognition site. The vector was then introduced in *E. coli* EC100 by chemical transformation. Digested pTM4 with *Ecl136II* was also further digested with *SacI* and used as negative control. Transformed cells were selected in LB with Cl 15 µg/ml (a chloramphenicol resistance gene is present in the backbone of the vector pTM4). Acquisition of the *tnp* module was confirmed by PCR-targeting the insert, with primers TNP1 and TNP2. The vector pTM4-*tnp* was then extracted from EC100 with a QIAGEN mini kit (QIAGEN, Germany), digested with *XmnI* and used to naturally transform the *A. baylyi* SD11 (Suc^S), introducing the *tnp* module into the ACIAD3309 gene. Selection of the transformed cells was done in LB with Suc 50 g/l and checked for loss of K^R (10 µg/ml).

Acquisition and the correct orientation of the insert in the acquired *A. baylyi* SD12 was checked with primers ACIAD3309-down-r + TNP1 and ACIAD3309-down-r + TNP2. Positive amplification with the first pair of primers and negative with the second one were indicative of the correct orientation of the *tnp* module (*tnpA* – *tnpR* – *tnpM*); this PCR was done with the Phusion polymerase.

A. baylyi KOC4

The non-competent *A. baylyi* KOC4 was constructed by deletion of the competence genes operon *comFECB*, by natural transformation of *A. baylyi* BD413 with DNA from a strain carrying a Δ *comFECB::dhfr* allele (trimethoprim resistant, W^R) [(Bacher et al., 2006), A. Utnes, unpublished data]. The transformed cells were selected in LB with W 250 µg/ml.

The correct acquisition of a non-competent *A. baylyi* isolate was checked by natural transformation using homologous DNA with a selective marker (DNA from *A. baylyi* SD2, K^R).

3. Other bacteria studied

Twenty-seven clinical *Acinetobacter* spp. isolates from the collection of the Faculty of Pharmacy of the University of Coimbra, which includes isolates from the University Hospitals of Coimbra, collected between 1992 and 1998, and from the Hospitals from Porto, gathered between 1995 and 1999, and 72 clinical and non-clinical samples (a kind gift from Prof. Dr. Maria José Saavedra), including 48 *Enterobacteriaceae*, 18 non-fermenters and six non-identified, isolated from domestic, wild and food-producing animals, collected in the Animal and Veterinary Science Centre from University of Trás-os-Montes e Alto Douro between 2006 and 2007, were screened for the presence of class 1 integron.

Additionally, clinical isolates of *A. baumannii* (strains 694, 695, 696 and 9043) and *Acinetobacter* spp. (strains 5227 and 5248) carrying a class 1 integron, isolated in 2001 and 2002 in Brazil (Mendes et al., 2007), were screened for the presence of MITEs (a kind gift from Dr. Mark Toleman).

4. Bacterial identification

Several methods were used when bacterial species identification was needed. These included the API20E and API20NE systems (BioMérieux, Marcy l'Étoile,

France), PCR detection of the *bla*_{oxa-51-like} gene, sequencing of the 16S rDNA and *rpoB* genes, and determination of the phenotypic characteristics of colonies on EMB (Eosin Methylene Blue) and MacConkey agar plates.

The API20E (for *Enterobacteriaceae* and other non-fastidious Gram-negative rods) and API20NE (for non-enteric Gram-negative rods) are commercial available systems that allow bacterial identification based on biochemical tests.

The EMB and the MacConkey agar media allow the growth of *Enterobacteriaceae*, while inhibiting the growth of Gram-positive bacteria. In the EMB agar medium, fermenters produce dark green colonies, and *E. coli* presents a characteristic metallic green sheen; weak fermenters, such as *Klebsiella* sp. and *Enterobacter* sp., grow as purple colonies; and non-fermenters, such as *Salmonella* sp., have transparent colonies. On the MacConkey agar, fermenters produce pink colonies and non-fermenters colorless colonies.

PCR-based detection of the *bla*_{oxa-51-like} gene was used to confirm the identification of *A. baumannii* species, as the gene is considered intrinsic to this species (Turton et al., 2006). Details of the amplification are explained in the PCR section (V, subsection 1.1).

Sequencing of the 16S rDNA and *rpoB* genes, both housekeeping genes, is further detailed in the below section describing DNA sequencing (section VI, subsection 1.4).

The Acinetobacter sp. 121FFC isolate was already known to belong to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (Da Silva, 2002). Further PCR-amplification of the *bla*_{oxa-51-like} gene and 16S rDNA sequencing revealed the species of this isolate to be *A. baumannii*. *Acinetobacter* sp. xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268 and 2276 were identified by detection of the *bla*_{oxa-51-like} gene and sequencing of the *rpoB* gene; these isolates also belonged to the *A. baumannii* species. This species name will be used throughout this thesis to refer to these isolates.

The Acinetobacter sp. 118FFC isolate was previously identified as *Acinetobacter* genomic species 10 by ARDRA (amplified ribosomal DNA restriction analysis) and AFLP (amplified fragment length polymorphism)

methods (Da Silva, 2002); recently the species name *A. bereziniae* was proposed (Nemec et al., 2010). The obtained sequence of the 16S rDNA was not detailed enough to determine the species of this isolate. However, the sequence of the *rpoB* gene confirmed that the isolate was *A. bereziniae*, and this species name will be used throughout this thesis for this strain.

The Brazilian *Acinetobacter* spp. strains 5227 and 5248 (Mendes et al., 2007) were identified to belong to the *A. baumannii* species by PCR-detection of the *bla*_{oxa-51-like} gene and sequencing of the *rpoB* gene. These strains will be mentioned as *A. baumannii* in this thesis.

The Api20E and API20NE systems and the media EMB and MacConkey were used in the identification of selected *Enterobacteriaceae* and non-fermenter bacterial isolates from animals. The species identification of these isolates will be used throughout this thesis for: *C. braakii* C7R376, *C. freundii* C16R385, *E. cloacae* C2R371, *E. cloacae* C8R377, *E. cloacae* C13R382, *E. coli* C9R378, *E. coli* C10R379, *E. coli* G30oviducto, *E. coli* G65intestino, *E. fergusonii* AS041A2 and *P. aeruginosa* Dog006M1.

II. Isolation of genetic material from bacteria

Different types of genetic material were isolated from the bacterial samples, depending on the assays where it was employed; and included total DNA, cell lysates and total RNA.

1. Total DNA

Total DNA, which includes chromosomal and plasmid DNA, was used in natural transformation assays and for DNA sequencing. Isolated plasmids were used in cloning procedures; in addition, plasmid extraction was used to screen for the presence of plasmids in donor, recipient and transformant bacteria.

Total DNA was isolated from ON cultures using Anion-Exchange Resin columns (Genomic DNA kit, QIAGEN, Germany), according to the manufacturer's protocol and resuspended in EB buffer, pH 8.5 (QIAGEN, Germany).

Plasmid DNA was also isolated from ON bacterial cultures by an alkaline lysis procedure, using Anion-Exchange Resin columns (Plasmid Mini/Midi Kit, QIAGEN, Germany), according to the manufacturer's instructions and resuspended in TE buffer (Tris-HCl, 10 mM; EDTA, 1 mM; pH 8.0), or Qiagen EB buffer.

Determination of DNA concentration

The concentration of the various DNA extracts was measured with a UV/VIS spectrophotometer (6405 Spectrophotometer, Jenway, England) in a 1 cm pathlength quartz cuvette or in Nanodrop ND-1000 (Nanodrop Technologies, USA). The DNA solution was measured at 260 nm and at 280 nm; the DNA is measured at 260 nm and the protein impurities at 280 nm. The concentration was calculated taking in consideration that 1 unit of OD equals 50 µg/ml of dsDNA (this was automatically calculated when using Nanodrop). The purity of the DNA was calculated by the ratio of OD_{260nm}/OD_{280nm} ; pure DNA has a ratio between 1.8 and 2.0.

Detection of plasmid DNA

Plasmid DNA was detected by agarose gel electrophoresis and ethidium bromide staining, as explained in section V, subsection 2.

2. Cell lysates

For the preparation of supernatants (lysate) of the heat-treated bacterial cell suspensions, 5 ml of ON cultures of the bacteria were centrifuged at $20\,000 \times g$ for 5 min and resuspended in MilliQ water (50 µl), followed by vortexing and heat treatment at 80 ° C for 15 min. The raw lysate was centrifuged at 13 000 rpm (Biofuge 13 or Biofuge pico, Heraeus Sepatech; 2-16P, Sigma) for 2 min,

and the supernatant containing DNA was collected (Nielsen et al., 2000). The lack of viable cells was confirmed by streaking aliquots on LB plates.

DNA used as template in PCR reactions was extracted in a similar way, with the exception that the cell suspension was heated at 100 °C for 10 min.

3. Total RNA

Total RNA was isolated from ON bacterial cultures using the NucleoSpin TriPrep kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Residual DNA was removed by treatment with rDNase (Macherey-Nagel, Germany), followed by RNA ethanol precipitation and resuspension in RNase-free water.

Ethanol precipitation of RNA was performed as follow: to 1 vol of sample was added 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of 100 % ethanol, followed by an incubation at - 20 °C for 2 hrs; centrifugation at 13 000 rpm in a tabletop centrifuge (Biofuge 13, Heraeus Sepatech) for 10 min; washing of the RNA pellet with 70 % ethanol and centrifugation at 13 000 rpm for 5 min; drying of the RNA pellet and resuspension in RNase-free water.

Determination of RNA concentration

The RNA concentration was measured with Nanodrop ND-1000 (Nanodrop Technologies, USA); 1 unit of OD at 260 nm equals 40 µg/ml of RNA (automatically calculated by the Nanodrop software). Pure RNA has a OD_{260nm}/OD_{280nm} ratio higher than 2.0.

III. Natural transformation assays

Natural transformation assays were performed on nitrocellulose membrane filters (filter transformation).

1. Filter transformation

Filter transformation was performed using an *in vitro* transformation protocol, as described by Ray and Nielsen (Ray and Nielsen, 2005). In this method, recipient bacteria are exposed to a donor DNA source on a nitrocellulose filter placed on agar-surfaces, followed by the application of selection to identify transformants from the recipient population (the procedure is summarized in Figure 4). Transformation reactions were done over 4 days, and each assay was performed in triplicate.

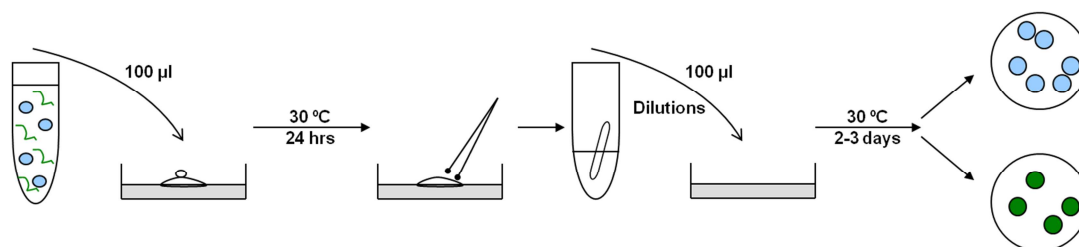


Figure 4. Schematic representation of the filter transformation procedure. The blue circles represent the recipient cells; the green circles the transformants; and the green lines, the donor DNA.

The competent cells were prepared as described below, and then used in the transformation experiments. Dominant selection of the transformants was done on agar plates with different antibiotics (see Table 2) and concentrations, according to the known or established resistance levels of the donors, as determined by resistance typing and MIC determination. All *A. baylyi* transformants were selected and grown on LB with R (25 µg/ml) plus: AM (25 µg/ml) for *A. baumannii* 65FFC transformants; CTX 10 or 50 µg/ml for *A. baumannii* 65FFC transformants; CN 10 µg/ml for *E. coli* K71-77, *K. pneumoniae* K66-45 and *P. aeruginosa* K34-73 transformants; K 10 µg/ml for *A. baumannii* 064 transformants; SC 10 µg/ml for *C. freundii* C16R385, *E. cloacae* C2R371, *S. enterica* serovar Rissen 486, *S. enterica* serovar Typhimurium 490 and *P. aeruginosa* SM transformants; and W 250 µg/ml for *E. coli* C10R379 and *E. fergusonii* AS041A2 transformants.

Filter transformation assays were done with:

i) the *A. baylyi* BD413 as recipient and total DNA extracted from various wild-type species (*A. baumannii* 064, *A. baumannii* 65FFC, *C. freundii* C16R385, *E. cloacae* C2R371, *E. coli* C10R379, *E. coli* K71-77, *E. fergusonii* AS041A2, *K. pneumoniae* K66-45, *P. aeruginosa* SM, *P. aeruginosa* K34-73, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490) or cell lysates (*A. baumannii* 064, *A. baumannii* 65FFC, *P. aeruginosa* SM, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490) as donor;

ii) the *A. baylyi* BD413 as recipient and total DNA extracted from class 1 integron-carrying *A. baylyi* transformants as donor (*A. baylyi* SD1, SD2, SD3, SD4, SD5 and SD6);

iii) the class 1 integron-carrying *A. baylyi* transformant SD2 as recipient and total DNA extracted from various wild-type class 1 integron-carrying species as donor (*A. baumannii* 064, *A. baumannii* 65FFC, *C. freundii* C16R385, *E. cloacae* C2R371, *E. coli* C10R379, *E. fergusonii* AS041A2, *P. aeruginosa* SM, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490);

iv) the class 1 integrase deletion carrying *A. baylyi* transformant (KOI) as recipient and total DNA extracted from various wild-type class 1 integron-carrying species (*A. baumannii* 65FFC, *P. aeruginosa* SM, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490) and from an *A. baylyi* transformant with a class 1 integron carrying an inactive integrase [KOI(Ps)1] as donor;

v) the class 1 integron-carrying *A. baylyi* *recA* deletion (RAM) recipient and total DNA extracted from various wild-type class 1 integron-carrying species as donor (*A. baumannii* 65FFC, *P. aeruginosa* SM, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490);

vi) the double mutant strain *A. baylyi* SD9 ($\Delta recBCD \Delta sbcCD$) as recipient and total DNA from *S. enterica* serovar Typhimurium 490 as donor;

vii) the competence mutant strain *A. baylyi* KOC4 ($\Delta comFECB$) as a recipient and total DNA from *A. baumannii* 064 as donor DNA.

viii) the *A. baylyi* SD12 (containing a *tnp* module) as recipient and total DNA from *A. baumannii* 65FFC as donor.

1.1 Preparation of competent cells

A 5 ml culture of the recipient (strains BD413, SD2, KOI, RAM, SD9, KOC4 or SD12) was prepared in LB plus antibiotic selection from one single colony, and incubated at 30 °C with good aeration (150 – 200 rpm) ON. The following day, an 1:100 dilution was done in a final volume of 25 ml LB (with antibiotic selection) and further incubated until cells reached the late exponential phase (Nielsen et al., 1997), $OD_{600nm} \approx 0.6$, which occurred after 5 to 8 hrs. The OD_{600nm} was correlated with the McFarland standard and the number of CFUs (colony-forming units)/ml, which allows the determination of the volume of buffer required to resuspend the cell pellet to a final concentration of approximately 10^9 cells/ml. The cells were pelleted at 4000 rpm for 10 min at 4 °C in a SS-34 rotor (Sorvall) or a IEC B-20A (Damon/IEC Division) centrifuge and the cell pellet was resuspended in the appropriate volume of cryostorage buffer (15 % [w/v] glycerol in LB). Aliquots of 1 ml were transferred into sterile 1.5 ml freezing tubes and placed immediately at - 80 °C. Each tube contained 10^9 cells/ml.

1.2 Experimental transformation

In the first day, using flame-sterilized forceps, 0.45 μ m sterile filters (Millipore GSWP4700 or Pall Corporation) were placed on the surface of LB agar plates, avoiding the formation of air bubbles.

The competent cells were thawed at room temperature and then centrifuged at 4000 rpm for 5 min in Biofuge 13, Biofuge pico (Heraeus Sepatech) or 2-16P (Sigma); the supernatant was discarded and the cell pellet resuspended in 800 μ l of sterile NaCl 0.85 % (w/v). Each 80 μ l contained 1×10^8 competent cells. Each transformation reaction was done with 10 μ g of donor DNA and 1×10^8 competent cells. A volume corresponding to 3 x 10 μ g of donor DNA was pipetted into sterile 1.5 ml Eppendorf tubes (if the total volume of donor DNA added was less than 60 μ l, sterile water was added until this volume); and 240 μ l (3 x 80 μ l) of competent cells were added and mixed by brief vortexing. In the negative control, 80 μ l of competent cells were added to 20 μ l of sterile water. 100 μ l of the donor DNA/recipient cells mixture were applied to the centre of each filter, and the plates incubated for 24 hrs at 30 °C.

On the second day, with the help of a flame-sterilized forceps, each filter was removed from the plate and placed in a cell resuspension tube with 4 ml NaCl 0.85 %; each tube was subsequently agitated on vortex to remove all the cells from the filter. Dilution series of each tube were done in 10-fold steps (from 10^{-1} to 10^{-7}) using previously prepared dilution tubes (0.9 ml of NaCl 0.85 %). To enumerate recipients, 100 μ l of the 10^{-6} and 10^{-7} dilutions were plated onto LBR/K/CI/W (depending on the recipient), and to enumerate transformants, 100 μ l of the undiluted and 10^{-1} - 10^{-4} dilutions were plated onto LBR/K/CI/W + appropriate selection-containing plates (depending on the donor). The cells were spread evenly using a flame-sterilized glass spreader or glass beads. After all the moisture was absorbed, the plates were incubated at 30 °C for 2-3 days.

After 2-3 days, when the colonies were large enough to enumeration, both recipient and transformant colonies were counted to obtain the number of CFUs per plate.

Controls

A positive control assay was set up to verify recipient cell competence and reproducible experimental conditions. It included transformation of *A. baylyi* BD413 by DNA from *A. baylyi* KTG, which contains a chromosomally located

nptII gene (K^R) (Nielsen et al., 1997; Ray et al., 2009). This control was performed once.

In each experimental transformation, a negative control was included, where the recipient cells were grown onto the filter with added water instead of the donor DNA and were streaked after 24 hrs on LBR/K/CI/W (depending on the recipient) plus selective antibiotic (depending on the donor). This allowed us to exclude the occurrence of mutants or the presence of contaminants.

1.3 Transformation frequency

The transformation frequency was calculated for each sample as the number of transformants per number of viable recipient cells. In some cases, the transformation frequency was also calculated as the number of transformants per unit amount of DNA; in these cases, the genome equivalent size was based on the average size calculated from published genomes of *A. baumannii* and *S. enterica* serovar Typhimurium, as available in GenBank (Apr. 2012) – 3.95 Mb and 4.99 Mb were considered, respectively.

IV. Antimicrobial susceptibility testing

The antimicrobial susceptibility determination was done to characterize donor, recipient and transformant bacteria. The disc diffusion method (Kirby-Bauer method) and the determination of the Minimal Inhibitory Concentration (MIC) by Etest were the methods performed. Both methods were performed and interpreted according to the CLSI (Clinical Laboratory Standards Institute) guidelines (CLSI, 2008), using Mueller-Hinton II (Fluka, BioChemika, Switzerland or Scharlau Chemie S.A., Spain) or PDM (AB Biodisk, Sweden) agar plates. A standard suspension (equivalent to 0.5 McFarland standard) of log phase cells in NaCl 0.85 % was prepared and inoculated onto the surface of the agar plates to form a lawn. Plates were incubated at 37 °C for 16-24 hrs.

1. Disc diffusion method

The disc diffusion method was used to compare the antimicrobial susceptibility profiles of donor and recipient bacteria with transformants, in order to detect isolates that might have acquired DNA resistance traits from the donor.

After the preparation and inoculation of the standard suspension of each bacterium, commercially available filter-paper discs containing known concentrations of antimicrobial agents were placed on the dried surface of the medium, accordingly with the CLSI guidelines (CLSI, 2008). After 18-24 hrs at 37 °C, the diameter of the zone of complete growth inhibition, if any, that had occurred around the disc was measured.

The antibiotics used for donor bacteria were mainly chosen based on the gene cassettes present in each bacterium's integron. The antimicrobial agents used to evaluate the transformants altered antimicrobial susceptibility were selected based on the resistance pattern of DNA donor bacteria given by the integron gene cassettes and the intrinsic resistance of the recipient bacteria. *A. baylyi* BD413 and other isolates used as recipient bacteria were tested for all antibiotics tested in donor and transformants bacteria. The antimicrobial discs used (Oxoid, England or AB Biodisk, Sweden) were: amikacin (AK; 30 µg), AM (10 µg), CTX (30 µg), ceftazidime (CAZ; 30 µg), CI (5 µg), compound sulphonamides (S3; 300 µg), CN (10 µg), imipenem (IMP; 10 µg), K (30 µg), meropenem (MEM; 10 µg), netilmicin (NET; 30 µg), R (5 µg), SC (100 µg), sulfadiazine (SD; 250 µg), streptomycin (SM; 10 µg), sulphamethoxazole/trimethoprim (SXT; 25 µg), W (5 µg), and tobramycin (TOB; 10 µg).

2. Minimal Inhibitory Concentration determination by Etest

When the antimicrobial susceptibility profile of transformants determined by the disc diffusion method showed significant changes when compared with the

recipient, the Etest method (AB Biodisk, Sweden) was used to quantify the MICs. MICs were measured twice, or, when in doubt of the exact MIC value, three times. MICs were also determined prior to the natural transformation experiments for both recipient and donor bacteria, to determine the appropriate concentration of antibiotics to be used in the transformants selection.

After the preparation and inoculation of the standard suspension of each tested bacterium, commercially available Etest strips containing a gradient of antibiotic concentrations were placed in the centre of the dried surface and incubated at 37 °C for 16-20 hrs. The MIC value was determined from the intersection of the edge of the inhibition ellipse with the side of the strip. The following Etests were used: AM, CTX, CAZ, CN, K, SC, sulphamethoxazole (SX), and TOB.

V. Polymerase Chain Reaction (PCR)

PCR was used to amplify different products, including resistance determinants and mobile elements. PCR-products were detected by agarose gel electrophoresis and ethidium bromide staining, and, when needed for further applications, were purified.

1. PCR-product amplification

In PCRs with a *Taq* DNA polymerase, the DNA template used was extracted as explained above in the production of cell lysates subsection (Section II, subsection 2). In amplification of products with a high-fidelity DNA polymerase, the DNA template was a suspension with a 1 µl loop sample of bacterial cells.

PCR amplification was performed in 0.2 ml tubes with a T-personal (Biometra, Göttingen, Germany), a MJ Mini (BIO-RAD, Portugal) or a PTC-200 (BIO-RAD, Norway) thermal cycler.

All primers used in this study are listed in Table 4 . Primers were design based on known sequences using the online programs Primer3 (<http://fokker.wi.mit.edu/primer3/input.htm>) and Oligoanalyzer (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>).

A few main rules were followed in the primers design for PCR and direct sequencing: length between 17 and 24 bp; 3' end finishing in G or C; no more than 4 repetitions of the same nucleotide in a row; self-dimer with a ΔG lower than - 4 Kcal/mole, and unintended dimer formation of the primer was preferred in the 5' than in the 3' end; hairpin with less than 5 aligned bases. When used in PCR reactions, the hetero-dimer between both primers should have a ΔG lower than - 4 Kcal/mole. All the rules were followed when possible; whenever not possible, a compromise between the parameters had to be done.

Table 4. Primers used in this study.

Primer	Sequence (5' – 3')	Reference
16S rDNA-F	AGAGTTTGATCMTGGCTCAG	(Weisburg et al., 1991)
16S rDNA-R	ACGGHTACCTTGTTACGACTT	(Weisburg et al., 1991)
16SF	GATCATGGCTCAGATTGAACG	(Starikova et al., 2012)
16SR	ACGACTTGACCCAGTCATC	(Starikova et al., 2012)
211F	CTGGGAGCACATCAACCTG	This study
211F1	GGAACAGGTAACAAGCCAATG	This study
211R	ATTGCCAGACCCGTGAATAC	This study
236F	CTAAAGGTTCCGCCACAAAC	This study
236F1	TGATGATTAGTGTCTGTTGACC	This study
236R1	GGCATTGGATTCGGTTTTATC	This study
3'-CS	AAGCAGACTTGACCTGA	(Levesque et al., 1995)
4311P	AGGTTTGGCGAAGTCGATG	This study
4311P2	CGGATGTTCCGACTATTTTCAGC	This study
4311P3	AACGAAGCCGCTATAAACCTC	This study
4311R	GAATACCAGCGACCCCTTG	This study
5'-CS	GGCATCCAAGCAGCAAG	(Levesque et al., 1995)
AACA7F	GGCTTAACCTCAGGCGT	(Da Silva, 2002)
AACC1F	AAGTTGGGCATACGGGAAG	This study
AACCR	CAGCAAGCGCGATGAATGTCT	(Da Silva, 2002)
AADA1R	GCCAAGCGATCTTCTTCTTG	This study
AADA2R	ACCAAGGCAACGCTATGTTT	This study
AADB1	ACGCAAGCACGATGATATTG	This study
AADB2	CGCAAGACCTCAACCTTTTC	This study
Ac1055F	GTGATAARATGGCBGGTCGT	(La Scola et al., 2006)
Ac1598R	CGBGCRTGCATYTTGTCRT	(La Scola et al., 2006)
BAK11w	AGTTTGATCMTGGCTCAG	(Bosshard et al., 2006)
BAK2	GGACTACHAGGGTATCTAAT	(Bosshard et al., 2006)
CAT1	CCCGCTTATTATCACTTATTCAGG	This study
CAT2	GCTGAACGGTCTGGTTATAGG	This study
CATF	GAAGTGATCTTCCGTCACAGG	This study
cat-f	CTCCGCTAGCGCTGATGTCC	This study

(continue)

Table 4 (continued)

Primer	Sequence (5' – 3')	Reference
cat-r	GTAGCACCAGGCGTTTAAGG	This study
CHRAR1	CAGCAAAAGGGGATGATAAG	This study
CS2	CGAATGGACAGCGAGGAG	This study
CS3	TCTCTACGACGATGATTTACACG	This study
CS486a	CTGACAGAAATGGGCTTTGAG	This study
CS490a1	TAGAGTCTTCCCTGACATTTTGC	This study
CS490b	TTCCTCCTGCTTATTCTGAGTTG	This study
CS490c	TGGAGGTTCTTCGGTCAAAC	This study
CS64b	TCCGTTTATGAGAGATACCAAATG	This study
CS64c	CGGTAATACTGTCTGGTGTTC	This study
CS64cF	GCGGTAAATCGTGGAGTGAT	This study
CS65a	CAAGGCAGTCAGTTTACCAGTG	This study
CS65a1	TCAGTCACAATGGAGGTGGA	This study
CS65b	GCAGCAATCGTCTGTTGG	This study
CS65bR	CGACCAACAATGGCAAGTC	This study
CS65c	GCATTGGCTAAATCTTATGAACG	This study
CS65cR	TTCCCAGCCATTTAGTTTGG	This study
CS65d	GACGAAGTACAAGCCAAAATACG	This study
DACCF	AGAAACTTGCCTCTTCAACTGG	This study
DACCR	ATCTTGCTCCTGCCTCTATGAC	This study
DownR	CGTCTTCAAGCATTTGAAGG	(Starikova et al., 2012)
FTSIF	TAACGACCTTGCGGATTTTC	This study
FTSIR	TGGCGTTTCTATCTGCTGTG	This study
GENAB	AACTTTGGCACTCTCAATACCC	This study
GENAB2	TGACAACGGCAAAGACCTTAC	This study
GENAB3	TCCATTTGTGCTTCTTTACGG	This study
GENAB4	CACGCCTGAATCCAATCTATG	This study
GENABR	CCCTGATGGCGGTCTTC	This study
GENABR2	CGGTAAAGGTGAGCAGAGG	This study
GENABR3	TCGTAGTCCGCATCACTAGC	This study
GENABR3R	AGCGACCCAATAGCCAATC	This study
GENABR4	CAGGATGATGCCGAGATTG	This study
GS1	GCCAGGACAGAAATGCCTCG	(Da Silva et al., 2002)
GS2	CAAAGACTCTGCTGCGGCAG	(Da Silva et al., 2002)
HS463a	CTGGATTTTCGATCACGGCACG	(Barlow et al., 2004)
HS464	ACATGCGTGTAATCATCGTGC	(Barlow et al., 2004)
INCINTF	TGATGCCTGCTTGTCTACG	This study
IS1	ACATTGCGCTTATTGGTATCG	This study
IS1F	GCAGTTCACTTACACCGCTTC	This study
IS2	ACCCTTCTTGAGGACAGTAGTTTG	This study
IS3	TCATCTGGTTTTGCGAATACTG	This study
ISAb1a	ATGCAGCGCTTCTTTCAGG	(Heritier et al., 2006)
ISAb1b	AATGATTGGTGACAATGAAG	(Heritier et al., 2006)
ISTA1	TGCTCAAGCCACACATTCA	This study
ISTA2	TTTTGTGCTCGGTGGAGAG	This study
ISTA3	AGCGGCATGAGGCACTAC	This study
ISTA4	GACCGATCTGACGGAAATACA	This study
ISTA5	GTATAAACCGTCCAAGAAATCGTC	This study
ISTA6	GTGCGGCGGAACCTTCTC	This study
ISTAA	GTCCAGGAACACCGTTTCG	This study
ISTAb	CGGGCAAACAGGTCAAAC	This study
MERA1	ATTCGCCGTCCCCTTCA	This study
MERC1	CGACACCCCAATCATCAAG	This study
MERCR	ACGAGGGACTGTTTCATCAGC	This study

(continue)

Table 4 (continued)

Primer	Sequence (5' – 3')	Reference
MERP1	TAGCGTGACGGTCTGGGTAG	This study
MERR1	TTTGAAAACCTGACCATTG	This study
MITE1	TGTGACTGACCATTAAAG	This study
MITE2	TGTCTTTGCACATTAAAG	This study
MITEP	ACCCAGATACGGCTGATGTC	This study
NCRINS	TGATTTAGTGTATGATGGTG	This study
orf5F	TGATATCGACGAGGTTGTGC	(Domingues, 2008)
orf5R	AAGTGTGCGACGTGGGTGAAT	(Domingues, 2008)
ORFXF	AGATGCGGTTATCGGGTTC	This study
OXA-51-likeF	TAATGCTTTGATCGGCCTTG	(Turton et al., 2006)
OXA-51-likeR	TGGATTGCACTTCATCTTGG	(Turton et al., 2006)
PBPAF	CAGTTGTGCGCAGCAGGTG	This study
PBPAR	ACTGATGAGCCGCTATGCTT	This study
PBPGF	CCACCACTGTCCGTAATGC	This study
PBPGR	GACAACACAGCGACCTGCT	This study
Ping0	ACTGTTTCGCCAGGCTCAAGG	Harms, unpublished
PONAF	TGGGTTTCTACGGCATGTATC	This study
PONAR	TCTTCGCCTGGTAAATCAGC	This study
PRMF	GCTTTGCTGTCTGCTGGTC	This study
PRMR	TCTGCTATGTGCTTCTGTTGC	This study
PSG	TACTCGCAGATCAACCACTTG	This study
qacEΔ1F	ATCGCAATAGTTGGCGAAGT	(Sandvang et al., 1998)
qacEΔ1R	CAAGCTTTTGCCCATGAAGC	(Sandvang et al., 1998)
recA-f	AGCAAGGCATTACAAGCTGC	This study
recA-r	AATTCTGTAGAAATCTGAGG	This study
recD-down-r	AACTTGATGTCACCAATGATCCACG	(Kickstein et al., 2007)
sbcC-r18	AAAAGGGATTTAAGCGTCCG	Harms, unpublished
sbcD-f11	ATGGTCATTATAGATATTC	Harms, unpublished
sul1B	GCAAGGCGGAAACCCGCGCC	(Sandvang et al., 1998)
sul1F	CTTCGATGAGAGCCGGCGGC	(Sandvang et al., 1998)
SURR	TGGAAAGTGGAGGCAACAG	This study
TET1	CCGCATTTGGCATTCTG	This study
TET2	ATTGCCGATATCACTGATGG	This study
TETA1	CGTCACCGCCCACTATG	This study
TETAA	GATCTGGTTCACTCGAACGAC	This study
TETAF	GCTGCCCTCTACTTGCTCTG	This study
TETR1	GAGAGCGGAGCCTGTTCA	This study
TETR2	CTTTTCGCCAATCCATCG	This study
TN1	CCCAAAGACCTCGGTAAGTTC	This study
TN1R	TTCTGGAAGTCTGCTGATG	This study
TN2	GACTTCGACGACTACCTGCTG	This study
TN3	TGACCTACGCCAAGCTGTC	This study
TN4	TGCACCTGCTAGGCTTCC	This study
TN4F	TTCCGAATTCTGCGGGCTCC	This study
TN5	CGGCTATCGTGCTGTGG	This study
TNIA1	TCGGCGTAGTAGTGGATGTG	This study
TNIA2	CCGCCACACCTTGTAG	This study
TNIA2R	GTTCTGACCAAGCAGAAGC	This study
TNIA3	CACGCCTTGTCTGGAATC	This study
TNIA4	CATCAGGCCGACGACTC	This study
TNIA5	ATGTTGCAGGCAGGAATAGC	This study
TNIA6	AGTTGCGATACCGTGTAGGC	This study
TNIAR	GGCTGTCACCCTCTGGGAAC	This study
TNIBa	ACGGCTACCACTCCTCAATCT	This study

(continue)

Table 4 (continued)

Primer	Sequence (5' – 3')	Reference
TNP1	GGGGTCGTCTCAGAAAACGG	This study
TNP2	ATGGAGGTGGTAGCCGAGGG	This study
TNPA26	TCAGCAAAGGGGATGATAAGT	This study
TNPA26A	GCAGGTGAAGTATCTCAATAACG	This study
TNPA26F	ACCAGCACCCAACCTAACCC	This study
TNPA26F1	TGTAAATGACGGTTAGACAG	This study
TNPAA1	ATCCGACATTACACATTCAACG	This study
TNPAb	TCTGGCAAAGTTCGAGGTG	This study
TNPAc	CTCGCCAGTTC AAGGACTTC	This study
TNPAc	CTCGCCAGTTC AAGGACTTC	This study
TNPAcR	CAGCAGTTCGGTGATCTTGA	This study
TNPAcR1	ATTCATCGACCTCCATCAGC	This study
TNPAd	ACGGACTTTCTACACCCATATCTC	This study
TNPAd	ACGGACTTTCTACACCCATATCTC	This study
TNPAe	CTTCTACCGATTGGGTGAAATC	This study
TNPAF1	GGTGATAACAAAGAATCGGTCAG	This study
TNPAg	CCGCCTTGGTGAAATCC	This study
TNPAg	CCGCCTTGGTGAAATCC	This study
TNPAi	CAGGCATTTGAGAAGCACAC	This study
TNPM2	CGGACGCTTGTGATTCCG	This study
TNPR1	TTCGGCAATACGCTCAGAC	This study
TNPRF1	TGGCGAACCTGATGCTGTCCG	This study
UpF2	AGTCTTCCCAGCCTGCA	(Starikova et al., 2012)
VS1	CTGGCTGCGTAGTTGTTTCA	This study
VS2	GGGCTGCGAGTTCAATAG	This study

Amplification with Taq DNA polymerase

In general, PCR assays were set up in two different mixtures:

- 25 µl final volume using 22.5 µl of the PCR SuperMix from Invitrogen (Alfagene, Portugal) (22 mM Tris-HCl, pH 8.4, 55 mM KCl, 1.65 mM MgCl₂, 220 µM of each dNTP, 22 U/ml recombinant *Taq* DNA Polymerase, stabilizers), 0.75 µl of each primer 10 µM and 1 µl of DNA (approx. 10 ng DNA).

- 50 µl final volume using 22.5 µl of the 2 x PCR MasterMix Dynazyme II from Finnzymes (Finnzymes, Finland) (0.04 U/µl DyNAzyme II DNA Polymerase, 20 mM Tris-HCl, pH 8.8 at 25°C, 3 MgCl₂, 100 mM KCl, stabilizers and 400 mM of each dNTP), 0.75 µl of each primer 10 µM, 25 µl of sterile water and 1 µl of DNA (approx. 10 ng DNA).

For some primers, the melting temperature (T_m) had to be calculated, to establish the annealing temperature (T_a) used in the PCR amplification program, using the formula (Sambrook and Russel, 2001):

$$T_m \text{ } ^\circ\text{C} = 2 \times (A + T) + 4 \times (G + C),$$

where $A + T$ = number of AT base pairs and $G + C$ = number of GC base pairs. The T_a used was 2-5 $^\circ\text{C}$ lower than the T_m .

The presence of the *bla*_{oxa-51-like} gene, class 1 integrons, class 1 integrase, gene cassettes and MITEs was determined by PCR using different primer sets and amplification conditions as described below.

Amplification with high-fidelity DNA polymerase

The reaction mix for amplification with the high-fidelity DNA polymerase Phusion (Finnzymes, Finland) included:

- 6 μl of buffer HF 5x, 0.75 μl of dNTPs 10mM each, 0.3 μl of each primer 100 μM , 0.3 μl of Phusion 0.02 U/ μl , 3 μl of DMSO 100 %, 1.2 μl of MgCl_2 50 mM, 1 μl of DNA (approx. 300 ng of DNA), and water to a final reaction volume of 30 μl .

The PCR program included an initial step at 98 $^\circ\text{C}$ for 30 sec, 30 cycles of 10 sec at 98 $^\circ\text{C}$, 10 sec at 58 $^\circ\text{C}$ and variable time at 72 $^\circ\text{C}$, depending on the size of the product to amplify (Phusion amplifies 1 kb per each 30 sec), and a last step at 72 $^\circ\text{C}$ for 30 sec.

The Phusion polymerase was used to amplify the PCR-products used in the cloning procedures, as explained above (section I, subsection 2.1).

1.1 PCR-based detection of the *bla*_{oxa-51-like} gene

The *bla*_{oxa-51-like} gene PCR detection was done for all the *Acinetobacter* spp. isolates with primers OXA-51-likeF and OXA-51-likeR (Turton et al., 2006). The transformants (AbII)4 and (AbII)L1 obtained from *A. baumannii* 064 in a previous work, and which acquired a β -lactamase (Domingues, 2008), were also subject to this PCR. *A. baumannii* 65FFC and *A. baylyi* BD413 were used as positive and negative control, respectively. The PCR program was run for 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 90 sec at 72 °C, and 10 min at 72 °C.

1.2 PCR-based detection of class 1 integrase

Class 1 integrase detection was performed in the wild-type bacteria submitted to the screen of class 1 integron presence; among the animal bacterial isolates, only those showing resistance or intermediate susceptibility to SXT were screened. Isolates with positive amplification of the class 1 integrase were then screened for classic class 1 integrons, as described in the following subsection (1.3).

The *intl1* gene was PCR-amplified with primers HS463a and HS464 (Barlow et al., 2004); the PCR program included an initial step at 94 °C for 7 min, 30 cycles at 94 °C for 30 sec, 56 °C for 1 min and 30 sec at 72 °C, followed by a final step at 72 °C for 10 min. Known integron-carrying and non integron-carrying bacteria were used as positive and negative controls, respectively.

1.3 PCR-based detection of class 1 integrons

The presence of class 1 integrons was assessed by PCR in the donor, recipient and transformant bacteria, as well as in the screen of class 1 integrons presence in the other wild-type bacteria that were not used as donor source in the transformation assays. The donor and recipient bacteria were used as

positive and negative controls, respectively, in class 1 integrons detection acquisition by the transformants. Known class 1 integron-carrying and non-class 1 integron-carrying bacteria were also used as positive and negative controls, respectively, in the screen of class 1 integrons.

Class 1 integrons were detected with a set of primers specific for the conserved regions, 5'-CS and 3'-CS (Levesque et al., 1995). The DNA amplification program consisted of an initial denaturation step (94 °C, 5 min), followed by 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 5 min), and a single final extension of 16 min at 72 °C (Da Silva et al., 2002).

1.4 PCR-based detection of gene cassettes

Isolates *A. baylyi* SD2, KOI and RAM contain an integron with the *aadB* gene cassette. The transformants obtained with these isolates as recipients, were checked for the presence of that gene cassette. *A. baylyi* BD413 was used as negative control, and each *A. baylyi* recipient was used as positive control in the PCRs in the respective transformants. PCR was performed with primers AADB1 and AADB2, and the amplification program consisted of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 90 sec at 72 °C, followed by 10 min at 72 °C.

Rearrangements of gene cassettes during integron transfer and transformants cultivation in the class 1 integrons with more than one gene cassette were tested by PCR, combining one primer binding in the 5'-CS region and one primer that binds in the distal gene cassette in the integron of the donor bacterium. The combination of primers changed depending on the donor bacterium: 5'-CS + AADA2R were used in *C. freundii* C16R385, *E. cloacae* C2R371, *P. aeruginosa* SM and *S. enterica* serovar Rissen 486 transformants; and 5'-CS + AADA1R were used in *E. fergusonii* AS041A2 and *S. enterica* serovar Typhimurium 490 transformants. A initial step of 5 min at 94 °C was followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, ending with 10 min at 72 °C.

1.5 PCR-based detection of MITEs

The 439 bp MITE-like structures (Gillings et al., 2009a) were screened by PCR in all the *Acinetobacter* spp. isolates (n = 34), using primers MITE1 and MITE2, for 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1.5 min at 72 °C, followed by a final step of 10 min at 72 °C. When present, the relative position of MITEs to the class 1 integron was confirmed with the pairs of primers MITE1 + CS3 and MITE2 + CS2 (CS3 and CS2 bind in the conserved regions of the class 1 integron, in an outwards direction), with the same program but with an extension time of 2 min, and adding DMSO 5 %. After detection of the MITE-like structure in *A. baumannii* 65FFC, by primer walking sequencing of the genetic context of the integron, this isolate was used as positive control; *A. baylyi* BD413 was the negative control.

2. PCR-product detection

All PCR amplification products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. The 0.7 or 1 % agarose gel prepared in 1 x TBE (Tris-borate-EDTA; Tris base 0.89 M, boric acid 0.89 M, EDTA solution 20 mM, pH 8.2) or 1 x TAE (Tris-acetate-EDTA; Tris base 0.4 M, acetic acid 0.2 M, EDTA solution 10 mM, pH 8.4) buffers, containing ethidium bromide (10 µg/ml), was run in 1 x TBE or TAE buffer, respectively, at 80 V and visualized in a UV transilluminator.

The samples were mixed in a 5:1 proportion with the loading buffer, bromophenol blue (5 x; glycerol 25 %; bromophenol blue 0.25 %, 10 ml). Different ladders were used according to the expected size of the products to be analysed and if quantification was needed: 1 Kb Plus DNA Ladder (Invitrogen), 19 lines from 100-12 000 bp; SmartLadder (Eurogentec), 14 lines from 200-10 000 bp; NZYDNA Ladder III (NZYTech), 14 lines from 200-10 000 bp; 50 bp DNA Ladder (Bioron), 10 lines from 50 to 700 bp.

3. PCR-product purification

When needed, purification of a PCR-product was done with one of the followed methods: QIAquick gel extraction kit (QIAGEN, Germany), QIAquick PCR purification kit (QIAGEN), or with the ExoSAP-IT (GE Healthcare, Norway, or USB Corporation, USA) PCR clean-up protocol.

The gel extraction and the PCR purification with QIAGEN kits were done according to the manufacturer's instructions.

In the ExoSAP-IT PCR clean-up protocol, 2 μ l of ExoSAP-IT were added per each 5 μ l of sample, followed by a PCR program with 30 min on 37 °C and 15 min at 80 °C. ExoSAP-IT could also be prepared adding 0.25 μ l of shrimp alkaline phosphatase 1 U/ μ l (USB Corporation, USA), 0.05 μ l of exonuclease I 10 U/ μ l (USB Corporation, USA) and water to 10 μ l.

VI. DNA sequencing

DNA sequencing of specific DNA fragments was done by direct sequencing of total DNA or PCR-products, and primer walking, using the BigDye 3.1 cycle sequencing terminator reactions (Applied Biosystems, USA) and an ABI3130XL genetic analyser, based on the manufacturer's instructions and on previously described protocols (Wang et al., 2000; Ray et al., 2009). Details are explained in the subsections below. This method is based on the Sanger system, where nucleotides incorporation detection relies on the dideoxynucleotide chain termination method; four sets of fragments are generated, one for each type of base, all with the same 5' end, and each with a base-specific 3' end (Sanger et al., 1977). Sequences were edited and aligned in the Sequencher v.4.2.2 program (GeneCodes, USA) and identified using the BLASTN program, available at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

The genome of three transformants was fully sequenced by the 454 pyrosequencing technology. Sequences were also identified with the BLASTN program.

1. PCR-product sequencing

PCR-products of the variable region of the class 1 integrons of 11 clinical isolates (*A. bereziniae* 118FFC, *A. baumannii* 121FFC, xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268 and 2276), and 11 animal-isolated bacteria (*C. braakii* C7R376, *C. freundii* C16R385, *E. cloacae* C2R371, *E. cloacae* C8R377, *E. cloacae* C13R382, *E. coli* C9R378, *E. coli* C10R379, *E. coli* G30oviducto, *E. coli* G65intestino, *E. fergusonii* AS041A2, *P. aeruginosa* Dog006M1) were sequenced. PCR-product sequencing of the 16S rDNA gene of *A. baumannii* 121FFC and *A. bereziniae* 118FFC, the *rpoB* gene from *A. bereziniae* 118FFC and *A. baumannii* xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268, 2276, 5227 and 5248, and genes coding PBPs in selected transformants were also done. Sequence of the MITEs was done in *A. bereziniae* 118FFC and *A. baumannii* 694, 695, 696, 5227, 5248 and 9043.

Before sequencing, the PCR-products were purified as described above (section V, subsection 3).

The sequencing reaction was performed in 20 µl of total volume, including 2 µl BigDye 3.1, 3 µl BigDye buffer 5 x, 3.2 µl primer 1 µM, 2 µl template and 9.8 µl of water. The PCR program run for 25 cycles for 3 min at 96 °C, 15 sec at 96 °C, 10 sec at 50 °C, and 4 min at 60 °C.

In some cases, sequencing was done by the company Macrogen (South Korea) or StabVida (Portugal).

1.1 Sequencing of class 1 integrons

All the class 1 integrons were amplified with the primers 5'-CS and 3'-CS as described above. Sequence of the variable region of the integron of one of the

non-fermenters and 10 of the *Enterobacteriaceae* isolated from animals was obtained with the primers 5'-CS and 3'-CS. For the other isolates, the sequence was obtained by primer walking, first with the primers 5'-CS and 3'-CS and then with designed primers, based on the sequence fragments obtained with the primers 5'-CS and 3'-CS: AACAF7 and AACCR for *A. bereziniae* 118FFC; AACCF1F, ORFXF and AADA1R for *A. baumannii* 121FFC, xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268 and 2276.

1.2 Sequencing of genes coding for PBPs

Transformants that did not acquire class 1 integron but showed reduced susceptibility to ampicillin were also screened for altered PBP-encoding genes. Primers for PBP genes were designed based on the published sequence of *A. baylyi* ADP1 (GenBank accession number CR543861) (Barbe et al., 2004): PBPGF and PBPGR for the *pbpG* gene (gene ID 2879431), which encode PBPs 7 and 8; PBPAF and PBPAR for the *pbpA* gene (gene ID 2879430), encoding PBP 2; FTSIF and FTSIR for the *ftsI* gene (gene ID 2878829) that encode PBP 3; DACCF and DACCR for the *dacC* gene (gene ID 2878457), encoding PBP 5; PRMF and PRMR for the ACIAD1184 gene (gene ID 2879963), encoding a putative PBP; and PONAF and PONAR for the *ponA* gene (gene ID 2878085), which encode PBP 1. The *dacC* gene was amplified in a PCR program constituted by 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 59 °C and 90 sec at 72 °C, and a final step at 72 °C for 10 min. The other PBP genes were amplified with the same program but with a T_a of 55 °C. *A. baylyi* was used as positive control. The same primers were used to sequence the respective genes.

1.3 Sequencing of MITEs

The PCR-products obtained from *A. bereziniae* 118FFC, *A. baumannii* 694, 695, 696, 5227, 5248 and 9043, with primers MITE1 and MITE2, as described before, were sequenced with both primers.

1.4 Sequencing of 16S rDNA and *rpoB*

Part of the 16S rDNA and the *rpoB* genes were sequenced to help in the species identification of some bacterial isolates. The PCR-products were amplified with the DNA polymerase Phusion, as explained in section V, subsection 1.

Identification of *A. baumannii* 121FFC by 16S rDNA amplification and sequencing was done with primers BAK11w and BAK2 (Bosshard et al., 2006). The 16S rDNA gene sequence of *A. bereziniae* 118FFC was not discriminative enough to determine the species of this isolate. Primers Ac1055F and Ac1598R were used to amplify and sequence the *rpoB* gene (La Scola et al., 2006) in the identification of the *A. bereziniae* 118FFC and *A. baumannii* xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268, 2276, 5227 and 5248.

2. Sequencing of integrons and their flanking regions

The genomic context of the class 1 integron in the donors *A. baumannii* 064, *A. baumannii* 65FFC, *P. aeruginosa* SM, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490 and in the class 1 integron-carrying transformants *A. baylyi* SD1, (St)1, (St)2, (SD1)1, (SD1)2, (SD1)3, SD2, (AbII)1, (AbII)2, (SD2)1, (SD2)2 and (SD2)3 was determined by direct sequencing and primer walking. The sequence of the conserved regions of the class 1 integron of *A. baumannii* 65FFC and *A. bereziniae* 118FFC, as well as the genomic context of the integron of this last isolate, were also determined by primer walking using total DNA as a substrate.

The total DNA was extracted as described before with a QIAGEN kit (section II, subsection 1). The sequencing reaction was performed in 20 µl of total volume, using 4 µl of BigDye 3.1, 2 µl of BigDye buffer 5 x, 4 µl of primer 10 µM, 1 µg of DNA and water to complete the volume. The PCR program consisted of 5 min at 95 °C and 99 cycles at 95 °C for 30 sec, 55 °C for 10 sec, and 60 °C for 4 min.

The first primers used in integron flanking regions, CS3 and CS2, were designed based on the conserved regions sequence of class 1 integrons (GenBank accession number M73819) and the other primers were designed based on the sequence previously obtained.

2.1 Class 1 integron flanking regions

A. baumannii 064 and respective transformants

To sequence the flanking region of the 5'-CS of the integron from *A. baumannii* 064 and integron-carrying transformants SD2, (AbII)1, (SD2)1, (SD2)2 and (SD2)3, the primers CS3, TNPAF1, TNPAc, TNPAcR1, TNPAb, TNPAc, TNPAAd, TNPAe, IS1, IS2, IS3, TETR1, TETR2, TETA1, TETAA, TET1, TET2, TETAf, GENABR, GENABR2, GENABR3, GENABR3R and GENABR4 were used.

The 3'-CS flanking region of *A. baumannii* 064 and SD2, (SD2)1, (SD2)2 and (SD2)3 was determined with the primers CS2, CS64b, CS64c, CS64cF, TNPA26, TNPA26F, TNPA26F1, GENAB, GENAB2, GENAB3 and GENAB4; primers CS2, CS64b, CS64c, 4311R, TNPA26, TNPA26A, 4311P, 4311P2 and 4311P3 were used in *A. baumannii* 064 and (AbII)1.

The complete flanking regions of the class 1 integron in transformant (AbII)2 was not determined; only primers TNPAF1 and GENABR4 were used to determine the sequence on the 5'-CS side and 4311P3 on the 3'-CS flanking region.

A. baumannii 65FFC

The 5'-CS flanking region in *A. baumannii* 65FFC was sequenced with primers CS3, CS65a1, CS65a, CS65cR, CS65c and CS65d.

The primers CS2, TNIAR, TNIABa and MITEP were used to establish the sequences neighbouring the 3'-CS of the class 1 integron.

A. bereziniae 118FFC

Primer CS3 was used to determine the region flanking the 5'-CS in *A. bereziniae* 118FFC.

The primers CS2, orf5F, CS65bR, TNIAR, CS65b, TNIBa and MITEP contribute to clarify the region surrounding the 3'-CS.

P. aeruginosa SM

Flanking regions of the 5'-CS of the class 1 integron from *P. aeruginosa* SM were sequenced with primers CS3 and TNPRF1.

The sequence of the contiguous region to the 3'-CS was attempted determined with primer CS2, but it was not possible to determine.

S. enterica serovar Rissen 486

The 5'-CS flanking region in *S. enterica* serovar Rissen 486 was determined with primers CS3, CS486a, TN1R, TN1, TN2, TN3, TN4, TN4F and TN5.

The region adjacent to the 3'-CS of the integron was sequenced with primers CS2 and CHRAR1.

S. enterica serovar Typhimurium 490 and respective transformants

The flanking region of the 5'-CS of *S. enterica* serovar Typhimurium 490 and class 1 integron-carrying transformants SD1, (St)1, (SD1)1, (SD1)2 and (SD1)3 were sequenced with the primers CS3, CS490a1, TNPAa1, TNPAcR, TNPAc, TNPAAd, TNPAg. Additionally, the primers 236F, 236F1 and PSG were also

used with transformants SD1, (SD1)1, (SD1)2 and (SD1)3, and 211F and 211F1 with transformant (St)1.

For the same isolates, sequences adjacent to the 3'-CS were determined with the primers CS2, CS490b, CS490c, ISTAa, ISTAb, ISTA1, ISTA2, ISTA3, ISTA4, ISTA5, ISTA6, TNIA1, TNIA2, TNIA2R TNIA3, TNIA4, TNIA5, TNIA6, MERA1, MERCR, MERC1, MERP1, MERR1. In addition, 236R1 and 211R sequenced regions in transformants SD1, (SD1)1, (SD1)2 and (SD1)3 and in (St)1, respectively.

The sequence determination in transformant (St)2 was only done from the borders of the Tn21, with primers MERR1 on the 3'-CS side and TNPR1, TNPA_d, TNPA_g, TNPA_i, CAT1, CAT2, CATF, NCRINS, IS1F on the 5'-CS surroundings. The same primers were also used in the 5'-CS flanking region of the *S. enterica* serovar Typhimurium 490.

2.2 Class 1 integron conserved regions

The conserved regions of the class 1 integron of *A. baumannii* 65FFC and *A. bereziniae* 118FFC were sequenced with the primers HS463a, HS464, GS1, GS2, qacEΔ1F, qacEΔ1R, sul1F and sul1B. The primers INCINTF and orf5R were also used in the sequencing of the *A. bereziniae* 118FFC isolate.

3. Full genome sequencing

Full genome sequencing was done by 454 pyrosequencing. This is a two step DNA sequencing system, based on the “sequencing by synthesis” principle, where the template DNA is fragmented, end-repaired, ligated to adapters, and clonally amplified by emulsion PCR. The amplification is linked with pyrosequencing in picolitre-sized wells. The detection of nucleotides incorporation relies on pyrophosphate (PPi) release and generation of a light signal (Voelkerding et al., 2009).

Full genome sequence of the transformants (St)3, (AbII)3 and (AbI)L2 was done by the 454 pyrosequencing technology at the Institute for Genome Sciences, School of Medicine, University of Maryland, Baltimore, USA. The sequence was done on the Roche/454 GS FLX Titanium platform, using one full plate and multiplexing of three 8 kb paired-end libraries. Between 137 080 and 283 658 sequence reads were generated per genome, resulting in single-scaffold assemblies with a length of 3 614 029 bp, 3 633 656 bp, and 3 667 429 bp and average sequencing depths of 13-fold, 18-fold, and 28-fold for (AbII)3, (AbI)L2, and (St)3, respectively. Sequence trimming, assembly, gene finding and annotation were performed with the automated CloVR-Microbe pipeline (Angiuoli et al., 2011b; Galens et al., 2011b), which is part of the Cloud Virtual Resource (CloVR) appliance (Angiuoli et al., 2011a) developed in the CloVR project (<http://clovr.org>). Briefly, raw sequence data were filtered and trimmed for quality and adaptor removal, and assembled with Celera Assembler (Miller et al., 2010). Gene predictions and functional annotations were carried out using the tools and decision process described in the IGS Standard Operating Procedure for Automated Prokaryotic Annotation (Galens et al., 2011a). The annotated assemblies resulted in between two and 20 scaffolds, i.e. one or more contigs bridged by paired-end reads. In each case, only one scaffold was larger than 10 000 bp. None of the smaller contigs showed significant sequence similarity to plasmid and/or phage sequences and were considered assembly artefacts.

Rearrangement detected in the transposon Tn21 of the transformant (St)3 was confirmed by PCR with primers SURR + TNPM2 and IS1F + MERR1. The PCR program included 94 °C for 7 min, 30 cycles of 30 sec at 94 °C, 1 min at 56 °C and 3 min at 72 °C, and a final extension step at 72 °C for 10 min.

VII. Pulse-field gel-electrophoresis (PFGE) and Southern blot hybridization

PFGE and Southern blot hybridization were done in order to identify the genomic location of class 1 integrons in the bacteria used as donors in natural transformation assays and in the transformants that acquired an integron in transformation of *A. baylyi* BD413 by the wild-type donor bacteria *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490.

1. PFGE

PFGE was performed based on a protocol previously described (Mugnier et al., 2010).

Incorporation of bacteria in agarose plugs

To prepare the DNA plugs, freshly ON grown cells were transferred into glass tubes with 2.5 ml suspension buffer (100 mM Tris, 100 mM EDTA; pH 8.0), in order to have a cell density of approx. 10^9 cells/ml. A volume of 1.5 ml of the suspension was transferred into Eppendorf tubes and centrifuged at 10 000 rpm for 5 min. The pellet was resuspended in 1 ml cold PIV-buffer (1 M NaCl, 10 mM Tris-HCl; pH 7.6). A volume of 50 μ l of lysozyme (10 mg/ml) was added to 450 μ l of the suspension and equilibrated at 50 °C. A volume of 500 μ l of 2 % low melting point agarose was added and the mixture was rapidly transferred to the plug mould.

Bacterial lysis

After solidification, the plug was placed in 1.5 ml of lysis buffer (Basic buffer – 100 mM EDTA, 6 mM Tris, 1 M NaCl, 0.5 % Brij 58 – 20 mg deoxycholate, 50 mg N-lauroylsarcosine, Na-salt, 10 mg lysozyme, 1 μ l RNase ONE [10 U/ μ l]) and incubated for 2 hrs at 37 °C, with slow shaking. The plug was then washed

in 1 ml of distilled water for 15 min. 1 ml of ESP buffer (0.5 M EDTA [pH 9.0 to 9.5], 1 % Sarkosyl) containing 50 µg/ml of proteinase K was added and incubated ON at 50 °C. Finally the plug was washed twice in 1 ml of TE buffer for 30 min and kept at 4 °C until used.

DNA restriction

The DNA agarose blocks were digested with the endonuclease I-CeuI (New England Biolabs, Beverly, MA), which specifically recognizes the rRNA operons (Liu et al., 1993). A thin slice of the plug was cut out and transferred to 1 ml of 1 M Tris-HCL (pH 7.5) and washed for 30 min, to remove the EDTA. The plug was incubated in 200 µl of NEBuffer 4 (New England Biolabs, Beverly, MA), containing BSA 100 µg/ml, at 37 °C for 30 min. The plug was then transferred into 200 µl of I-CeuI master mix (containing NEBuffer 4 10 x, BSA 100 µg/ml, 3.35 U of I-CeuI enzyme and water) and incubated at 37 °C for 3 hrs. Finally, the plug was washed twice for 30 min in 200 µl 0.5 x TBE buffer.

Separation of the restricted fragments

The I-CeuI fragments were separated in a 1 % agarose gel by PFGE using a CHEF-DR III apparatus (Bio-Rad, Hercules, Calif.) at 15 °C, 6 V/cm with pulse time ramped from 20 to 120 sec over 11 hrs, followed by ramping from 60 to 100 sec for 11 hrs [adapted from Liu *et al.*, (Liu et al., 1993)]; the Lambda PFG ladder (New England Biolabs, Beverly, MA) was used as standard marker.

After this, the gel was stained in ethidium bromide-solution for 40 min, de-stained in distilled water-solution for 50 min, and visualized in an UV transilluminator.

2. Southern blot

The separated DNA was transferred by vacuum blotting (VacuGene XL, Pharmacia Biotech) to a positively charged nylon membrane (Roche, Germany), as described by Sambrook *et al.* (Sambrook et al., 1989). The

membrane was placed in the equipment and the gel was positioned on top of it. The frames were tightened in order to create vacuum, and the vacuum pump was set to work at 50 mbar during the blotting. The depurination solution (0.25 M HCl) was added, maintained for 30 min and then removed. The denaturation solution (1.5 M NaCl and 0.5 M NaOH) was added, kept for 40 min and removed. The neutralization solution (1.0 M Tris-Base and 1.5 M NaCl) was added and incubated for 30 min. Finally, the transfer solution (20 x SSC – 175.3 g of NaCl and 88.2 g of sodium citrate in 1 ml, pH 7.0) was added and incubated for 90 min. The gel was removed and the membrane was rinsed in the transfer solution for 10 min and then dried for 30 min. The DNA was covalently bound to the membrane by UV-crosslinking (150 mJoule). The membrane was washed in distilled water, air-dried and stored at 4 °C until used.

3. Probe hybridization

The 16S rDNA probe was the first to be used in hybridization, followed by the hybridization of the probe *intl1*.

Preparation of DIG-labelled probes

The 16S rDNA and *intl1* probes were amplified by PCR with primers 16S rDNA-F and 16S rDNA-R (Weisburg et al., 1991) and HS463a and HS464 (Barlow et al., 2004), respectively, and labelled with a PCR digoxigenin (DIG) probe synthesis kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

The PCR MasterMix included 5 µl of PCR DIG Probe Synthesis Mix 10 x, 5 µl PCR buffer with MgCl₂ 10 x, 1 µl of each primer 50 µM, 1 µl of enzyme and water to 44 µl; to this mix was added 6 µl of the DNA template (a purified PCR-product obtained with the same pair of primers). The PCR program included an initial step at 96 °C for 5 min, followed by 30 cycles at 96 °C for 30 sec, 53 °C for 30 sec and 1 min at 72 °C, and a final extension step of 10 min at 72 °C.

The amplified labelled probe was run in a 1 % agarose gel electrophoresis and recovered by gel extraction (QIAGEN gel extraction kit, Germany), according to the manufacturer's instructions. The probe concentration was estimated using a standard detection procedure (explained below) and a control DNA of known concentration. A good probe concentration in the hybridization solution was considered to be approx. 25 ng/ml; the probes were frozen at - 20 °C until used.

DIG-labelled probe hybridization

Hybridization of the probes was done at 68 °C; detection was performed with a DIG luminescent detection kit (Roche).

In a hybridization tube, 30 ml of pre-warmed prehybridization solution (5 x SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 1 % blocking solution [5 g blocking reagent in 500 ml of maleic acid buffer – 0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) were added to the membrane and incubated in the hybridization oven (Hybaid) for 2 hrs. Meanwhile, the hybridization solution (probe plus prehybridization solution) was boiled, to denature the probe, replaced the prehybridization solution and was incubated ON. On the next day, 2 washes for 5 min were done with 100 ml of 2 x stringency buffer (2 x SSC and 0.1 % SDS) at 68 °C; 2 more washes of 15 min each followed with 100 ml of 0.5 x stringency buffer, also at 68 °C.

Immunological detection of the DIG nucleotides

The immunological detection of the DIG nucleotides was performed according to the manufacturer's instructions. The membrane was first rinsed for 5 min in wash buffer (maleic acid buffer and Tween 20 0.3 %) at room temperature. Incubation in 100 ml of 1 x blocking solution for 30 min at room temperature followed. Incubation in 30 ml of a 1:10 000 dilution of anti-DIG-AP conjugate in blocking solution was done for 30 min. After this, 2 washes of 15 min each in 100 ml of wash buffer and equilibration of the membrane in 30 ml of detection buffer (0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5) for 5 min were done. The membrane was then transferred to the reaction bag and added 1 ml of CSPD;

after 5 min of incubation, excess of solution was removed and the bag was sealed. The membrane was protected from the light and incubated at 37 °C for 15 min. The film was developed using Lumi-film Chemiluminescent detection film in the automatic developer, after an exposure time of 15 min (exposure time could be extended when the luminescence was weak).

Stripping of the membrane

After the hybridization of the first probe, the 16S rDNA, stripping of the membrane was conducted to allow the hybridization of the second probe, *intl1*. First, the membrane was rinsed in water and then washed 2 times for 15 min in pre-warmed stripping solution (0.2 M NaOH and 0.1 % SDS) at 37 °C. Finally, it was washed in 2 x SSC, twice for 15 min. Pre- and hybridization with the second probe was done afterwards.

VIII. Reverse-transcriptase PCR (RT-PCR)

RT-PCR was performed to detect the expression of the class 1 integrase gene, *intl1*, present in the class 1 integron of the transformant *A. baylyi* SD2.

Class 1 integrase gene expression in RNA was included in the PCR analyses to verify absence of DNA, and PCR targeting the 16S rDNA genes was used to confirm successful complementary DNA (cDNA) synthesis.

Reverse transcription was performed with 1 µg of total RNA as template, extracted as described in section II, subsection 3, using the MonsterScript 1st-Strand cDNA synthesis kit with random 9-mer primers (Epicentre, USA), according to manufacturer's instructions. 1 µg of total RNA sample was added to 2 µl of random 9-mer primers (50 µM) and RNase-free water up to 15 µl. This mix was incubated at 65 °C for 1 min, then 1 min on ice, followed by a brief centrifugation. A volume of 4 µl of MonsterScript 5 x cDNA PreMix and 1 µl of MonsterScript Reverse Transcriptase were added, and the mix was incubated at 37 °C for 5 min. Finally, it was placed in the thermal cycler for 5 min at 42 °C,

40 min at 60 °C and 5 min at 90 °C. The PCR-product was chilled on ice for at least 1 min and 2 µl of the resulting cDNA was used in end-point PCR reactions, as described before. Primers HS464 and HS463a (Barlow et al., 2004) amplified a 473 bp fragment of *intl1*, and primers 16SF and 16SR (Starikova et al., 2012) an approx. 1500 bp fragment of 16S rDNA. The PCR-products were detected by agarose gel electrophoresis, as explained in section V, subsection 2.

IX. Biological fitness measurements

The relative fitness (W) of class 1 integron-carrying transformants was estimated by pairwise competition experiments between the transformants and the untransformed recipient strain:

- i) *A. baylyi* BD413 vs transformant *A. baylyi* SD3 (*bla*_{IMP-5}-carrying integron), selection with CTX 20 µg/ml, 23 replicates;
- ii) *A. baylyi* BD413 vs transformant *A. baylyi* (St)3 (*bla*_{OXA-30}-*aadA1*-carrying integron), selection with SC 10 µg/ml + AM 5 µg/ml, 24 replicates;
- iii) *A. baylyi* BD413 vs transformant *A. baylyi* (SD1)1 (*bla*_{OXA-30}-*aadA1*-carrying integron), selection with SC 10 µg/ml + AM 5 µg/ml, 19 replicates;
- iv) *A. baylyi* BD413 vs transformant *A. baylyi* SD4 (*bla*_{OXA-30}-*aadA1*-carrying integron), selection with SC 10 µg/ml + AM 5 µg/ml, 20 replicates;
- v) *A. baylyi* BD413 vs transformant *A. baylyi* SD5 (*dfrA12*-*aadA2*-carrying integron), selection with SC 20 µg/ml + W 250 µg/ml, 21 replicates;

vi) *A. baylyi* BD413 vs transformant *A. baylyi* SD6 (*aacA4-bla_{PSE}-aadA2*-carrying integron), selection with SC 20 µg/ml + AM 50 µg/ml, 20 replicates;

vii) *A. baylyi* BD413 vs transformant *A. baylyi* SD2 (*aadB*-carrying integron), selection with K 25 µg/ml, 23 replicates;

viii) *A. baylyi* BD413 vs transformant *A. baylyi* (SD2)1 (*aadB*-carrying integron), selection with K 25 µg/ml, 23 replicates.

The competition assays were performed in S2-minimal medium with lactic acid (1.5 g KH₂PO₄, 13.5 g Na₂HPO₄, 0.1 g MgSO₄, 2 g NH₄Cl, 1 ml [1 % solution] CaCl₂, 0.5 ml [0.1 % solution] FeSO₄·7H₂O; 1.5 ml lactic acid [20 mM]; pH 6.7; to 1000 ml), for 24 hrs, as previously described (Ray et al., 2009). Each competition was performed with independent starting cultures. The protocol is represented in Figure 5.

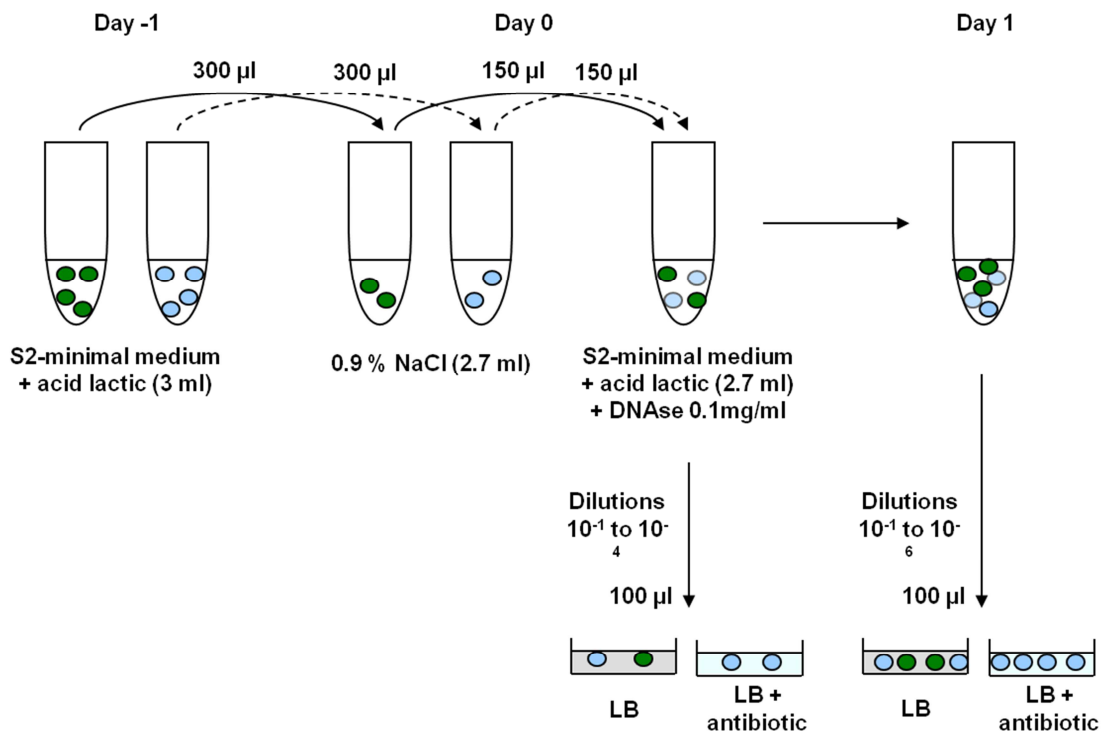


Figure 5. Schematic representation of the pairwise competition procedure. The green circles represent the competitor A and the blue circles the competitor B.

A single CFU from a fresh plate was inoculated in 3 ml of pre-warmed medium, and incubated at 37 °C ON with good aeration (225 rpm) (Day -1). On the next day (Day 0), cultures were diluted 1:10 in 0.9 % NaCl, and equal amount (150 µl) of each competitor was transferred into pre-warmed medium supplied with 0.1 mg/ml DNase (to avoid the possibility of natural transformation). To determine the initial cell density [A(0) and B(0)], 100 µl were taken and diluted up to 10⁻⁴ in 0.9 ml of NaCl 0.9 %; 100 µl of different dilutions were plated in antibiotic-free LB and in LB supplemented with the selective antibiotic, and incubated at 37 °C for 48 hrs. The competition culture was then incubated at 37 °C with agitation at 225 rpm. After 24 hrs, the competition assay was stopped (Day 1); the culture was diluted in NaCl 0.9 % up to 10⁻⁶ and 100 µl of different dilutions were plated in LB with antibiotic selection and without selection, to calculate the final density [A(1) and B(1)] of each competitor, and incubated at 37 °C for 48 hrs.

CFUs in plates from Day 0 and Day 1 were counted. The cell density of the integron-containing isolates were given by the number of CFUs in LB with antibiotic selection and the cell density of *A. baylyi* BD413 was calculated subtracting the number of colonies on the LB + antibiotic plate from the LB plate.

The W was calculated as the ratio of the Malthusian (M) parameter of each competitor:

$$W_{AB} = M_A/M_B,$$

where $M_A = \ln [A(1)/A(0)]$, $M_B = \ln [B(1)/B(0)]$, A(0) = Estimated density of A at Day 0 (cells per ml), B(0) = Estimated density of B at Day 0 (cells per ml), A(1) = Estimated density of A at Day 1 (cells per ml), and B(1) = Estimated density of B at Day 1 (cells per ml).

The mean, the standard deviation and the statistical significance of differences in W were calculated with SPSS (vs. 17) software.

Results

I. Characterization of wild-type bacteria

Wild-type bacteria were identified and screened for the presence and composition of some MGEs, as well as associated antimicrobial resistance and the genetic environment of such MGEs.

1. Bacterial identification

A few isolates were identified to the species level using a variety of methods. *Acinetobacter* sp. 121FFC was previously known to belong to the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex (Da Silva, 2002). The positive amplification of the *bla*_{oxa-51-like} gene by PCR in this study strongly indicated that this isolate was an *A. baumannii*. Sequencing of the 16S rDNA gene confirmed this species identification.

PCR amplification of the *bla*_{oxa-51-like} gene was positive in *Acinetobacter* spp. xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268, 2276, 5227 and 5248 isolates. This result, together with the sequence of the *rpoB* gene allowed to identify these isolates as belonging to the *A. baumannii* species.

Acinetobacter sp. 118FFC was previously identified as *A. bereziniae* [formerly *Acinetobacter* genomic species 10 (Nemec et al., 2010)] by ARDRA and AFLP methods (Da Silva, 2002). The absence of the *bla*_{oxa-51-like} gene and the sequence of the *rpoB* gene as observed in this study confirmed the species identification.

Observations of phenotypic traits in selective media were used first to presumptively identify the bacteria isolated from animals, and the Api20E and API20NE systems were subsequently used to confirm the species of some of the isolates studied. Among the *Enterobacteriaceae*, *C. braakii* C7R376, *C. freundii* C16R385, *E. cloacae* C2R371, *E. cloacae* C8R377, *E. cloacae* C13R382, *E. coli* C9R378, *E. coli* C10R379, *E. coli* G30oviducto, *E. coli*

G65intestino, and *E. fergusonii* AS041A2 were identified to the species level, as well as the non-fermenter *P. aeruginosa* Dog006M1.

2. Class 1 integrase and integron detection and composition

The detection of class 1 integrase and integrons was done by PCR. The composition of the variable region of the class 1 integrons detected was determined by DNA sequencing. Sequencing of the integrase gene in some isolates also allowed the determination of the versions of the gene cassette promoters present.

2.1 Donor bacteria

In order to be able to determine the genetic integrity of the integrons from the donor genomes after transformation of *A. baylyi*, the presence and composition of all the integrons in the donor genomes was assessed by class 1 integron-specific PCR, and by DNA sequencing by primer walking (listed in Table 2). Moreover, the genomic context of the class 1 integron was determined in some donor bacteria. PCR with primer pairs specific for the amplification of class 1 integron variable region yielded a single product, which in all cases, upon sequencing, contained at least one known antibiotic resistance gene. The opportunity to directly sequence each PCR-product without further cloning indicated the presence of only a single type of integron in each donor genome. In *C. freundii* C16R385 and in *E. cloacae* C2R371, the integron harboured a central region of 1913 bp, including the gene *dfrA12* (CDS 498 bp), which is known to encode a dihydrofolate reductase type I responsible for trimethoprim resistance, the *orfF* gene (CDS 291 bp) encoding a hypothetical protein, and the *aadA2* gene (CDS 792 bp) encoding an aminoglycoside adenylyltransferase related to streptomycin and spectinomycin resistance.

The amplification of the variable region of class 1 integron in *E. coli* C10R379 yielded a region with 769 bp, containing the *dfrA7* gene (CDS 474 bp) encoding a dihydrofolate reductase type I responsible for trimethoprim resistance.

The variable region of *E. fergusonii* AS041A2 was 1594 bp long and contained the gene *dfrA1* (CDS 474 bp), which encodes a dihydrofolate reductase type I responsible for trimethoprim resistance, and the *aadA1* gene (CDS 792 bp) encoding an aminoglycoside adenylyltransferase related to streptomycin and spectinomycin resistance.

2.2 Recipient bacterium

The recipient strain *A. baylyi* BD413 does not carry identifiable integrons (Barbe et al., 2004), as also confirmed in this study by class 1 integrons-targeting PCR in the strain BD413 and genome and direct DNA sequencing of transformants. PCR targeting the class 1 integrase and gene cassettes also showed absence of these elements in the recipient *A. baylyi* BD413.

2.3 Other bacteria

PCR targeting the class 1 integrase was positive in 34.3 % of the screened samples (34/99); and were further subject of PCR for class 1 integron.

Among the 99 isolates screened, 18 (18.2 %) were positive for class 1 integron (Table 5); thus 59 % (18/34) of the isolates with an amplifiable class 1 integrase were also PCR positive for a class 1 integron. Taking in consideration the origin of the isolates, 40.7 % (11/27) of the *Acinetobacter* spp. contained a class 1 integron, and these included the *A. baumannii* 121FFC, xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268 and 2276, and the *A. bereziniae* 118FFC. Amongst the bacteria of animal origin, 9.7 % (7/72) carried a class 1 integron: *C. braakii* C7R376, *E. cloacae* C8R377, *E. cloacae* C13R382, *E. coli* C9R378, *E. coli* G30oviducto, *E. coli* G65intestino and *P. aeruginosa* Dog006M1. If we take into

consideration the 4 isolates also collected from animals that were used as donor DNA, class 1 integrons were present in 14.5 % (11/76) of the isolates.

Table 5. Bacterial isolates carrying class 1 integron with the respective gene cassettes and expected resistance pattern.

Bacteria	Origin	Integron size (bp) ^a	Gene cassettes	Integron-associated resistance
<i>A. baumannii</i> 121FFC	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> xx29	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 131	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 1778	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 1783	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 1873	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 1989	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 2267	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 2268	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. baumannii</i> 2276	Clinical	2542	<i>aacC1-orfA-orfB-aadA1</i>	CN, SC, SM
<i>A. bereziniae</i> 118FFC	Clinical	2229	<i>aacA7-bla_{VIM-2}-aacC1</i>	AK, CN, NET, TOB, BLAC
<i>C. braakii</i> C7R376	Rabbit	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>E. cloacae</i> C8R377	Rabbit	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>E. cloacae</i> C13R382	Rabbit	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>E. coli</i> C9R378	Rabbit	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>E. coli</i> G30oviducto	Chicken	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>E. coli</i> G65intestino	Chicken	1913	<i>dfrA12-orfF-aadA2</i>	SC, SM, W
<i>P. aeruginosa</i> Dog006M1	Dog	1336	<i>aadA6-orfD</i>	SC, SM

^a Obtained with 5'-CS and 3'-CS primers (Levesque et al., 1995).

AK – amikacin; BLAC – β -lactams; CN – gentamicin; NET – netilmicin; SC – spectinomycin; SM – streptomycin; TOB – tobramycin; W – trimethoprim.

The class 1 integron of *A. baumannii* 121FFC, xx29, 131, 1778, 1783, 1873, 1989, 2267, 2268 and 2276, revealed a variable region of 2542 bp, containing four gene cassettes, the *aacC1* (CDS 534 bp) encoding an aminoglycoside acetyltransferase responsible for gentamicin resistance, the *orfA* (CDS 513 bp) and the *orfB* (CDS 306 bp) both encoding hypothetical proteins, and the *aadA1* (CDS 792 bp).

The class 1 integron of *A. bereziniae* 118FFC revealed a variable region of 2229 bp, which included three gene cassettes, the *aacA7* (CDS 459 bp)

encoding an aminoglycoside acetyltransferase, which confers resistance to amikacin, netilmicin and tobramycin, the *bla*_{VIM-2} gene (CDS 801 bp), responsible for the β -lactamase VIM-2 production related with β -lactams resistance, and the *aacC1* gene (CDS 465 bp). This gene cassette array was identified for the first time, and the number In796 was attributed by the INTEGRALL database of class 1 integrons; the nucleotide sequence was deposited in the GenBank database with the accession number JX235356.

The isolates *C. braakii* C7R376, *E. cloacae* C8R377, *E. cloacae* C13R382, *E. coli* C9R378, *E. coli* G30oviducto and *E. coli* G65intestino were determined to carry a class 1 integron with the same gene cassette array. This array is also the same as the one detected in isolates *C. freundii* C16R385 and in *E. cloacae* C2R371, used as donor source: *dfrA12-orfF-aadA2*. Resistance to trimethoprim, streptomycin and spectinomycin is associated with this gene cassette array.

The variable region of *P. aeruginosa* Dog006M1 was 1336 bp long and included two gene cassettes, the *aadA6* (CDS 846 bp) encoding an aminoglycoside adenyltransferase associated with streptomycin and spectinomycin resistance, and the *orfD* gene (CDS 279 bp) that encodes a hypothetical protein.

2.4 Gene cassette promoters

Sequence of the integrase gene of the class 1 integron present in some of the bacteria allowed us to determine the P_c and P2 promoter versions present (Table 6). The class 1 integron of *A. baumannii* 064, *A. baumannii* 65FFC and *A. bereziniae* 118FFC carried the strong version of the P_c promoter, P_cS, together with an inactive version of the P2 promoter. In the class 1 integron of *S. enterica* serovar Typhimurium 490 the weak version of the P_c, P_cW, and the active version of P2 were present. The TGN-10 extension, which result in increased promoter efficiency (Jove et al., 2010), was not found in these four promoters. The combinations of the P_c and P2 variants are similar to what is frequently found in other bacteria. The P_cW and P_cS are usually associated with an active and an inactive version of P2, respectively (Jove et al., 2010).

Table 6. P_c and P2 promoter sequences found in class 1 integron-containing bacteria in this study.

Bacteria	P _c			P2		
	-35 sequence	Spacer length (bp)	-10 sequence	-35 sequence	Spacer length (bp)	-10 sequence
<i>A. baumannii</i> 064	TTGACA	17	TAAACT	TTGTTA	14	TACAGT
<i>A. baumannii</i> 65FFC	TTGACA	17	TAAACT	TTGTTA	14	TACAGT
<i>A. bereziniae</i> 118FFC	TTGACA	17	TAAACT	TTGTTA	14	TACAGT
<i>S. enterica</i> Typhimurium 490	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT

3. Detection of plasmids

The presence or absence of plasmids in the donor and recipient bacteria was determined by plasmid extraction with a QIAGEN kit, followed by separation by agarose gel electrophoresis. Carriage of plasmids was detected in *A. baumannii* 064, *C. freundii* C16R385, *E. cloacae* C2R371, *E. fergusonii* AS041A2, *S. enterica* serovar Rissen 486 and *S. enterica* serovar Typhimurium 490. Plasmids were not detected in *E. coli* C10R379. *A. baumannii* 65FFC (Da Silva et al., 2002) and *P. aeruginosa* SM (Caetano et al., 2007) are known to not carry a plasmid. Plasmids were not detected in the recipient *A. baylyi* BD413 as well. PFGE and Southern blot hybridization with the 16S rDNA probe also supported these results.

4. Genomic location of the class 1 integrons

The genomic location of the integrons was determined only in the bacteria used as donor source in the natural transformation assays, by PFGE and Southern blot hybridization and, in some cases, by DNA sequencing.

4.1 PFGE and Southern blot hybridization

The genomic location of the class 1 integrons in the donor genomes was determined by PFGE and Southern blot hybridization. The endonuclease I-*CeuI*, which specifically recognizes the rRNA operons, was used to digest the genome of the isolates. Hybridization of the probes on the digested fragments separated by PFGE was interpreted as follow: co-hybridization of the *intl1* and 16S rDNA probes indicated chromosomal location, whereas hybridization only with the *intl1* probe was interpreted as indicating a plasmid location of the class 1 integron. It is known that *A. baylyi* has seven rRNA operons, producing seven fragments after digestion with I-*CeuI* (Gralton et al., 1997); indeed seven bands were seen in BD413 after the PFGE (Figure 6A). The lack of additional bands supported the absence of plasmids in this strain. *A. baylyi* BD413 was used as a negative control for the reliability of the results of co-hybridization of the probes, as class 1 integrons are absent from its genome. A plasmid location of the class 1 integron was shown in the donors *S. enterica* serovar Typhimurium 490, *E. cloacae* C2R371 and *C. freundii* C16R385, while class 1 integrons in the *A. baumannii* 064, *S. enterica* serovar Rissen 486 and *E. coli* C10R379 were located on the chromosome (Figure 6). *A. baumannii* 65FFC (Da Silva et al., 2002) and *P. aeruginosa* SM (Caetano et al., 2007) are known to carry the class 1 integron in the chromosome. Repeated PFGE was not conclusive for the determination of the location of the integron in *E. fergusonii* AS041A2.

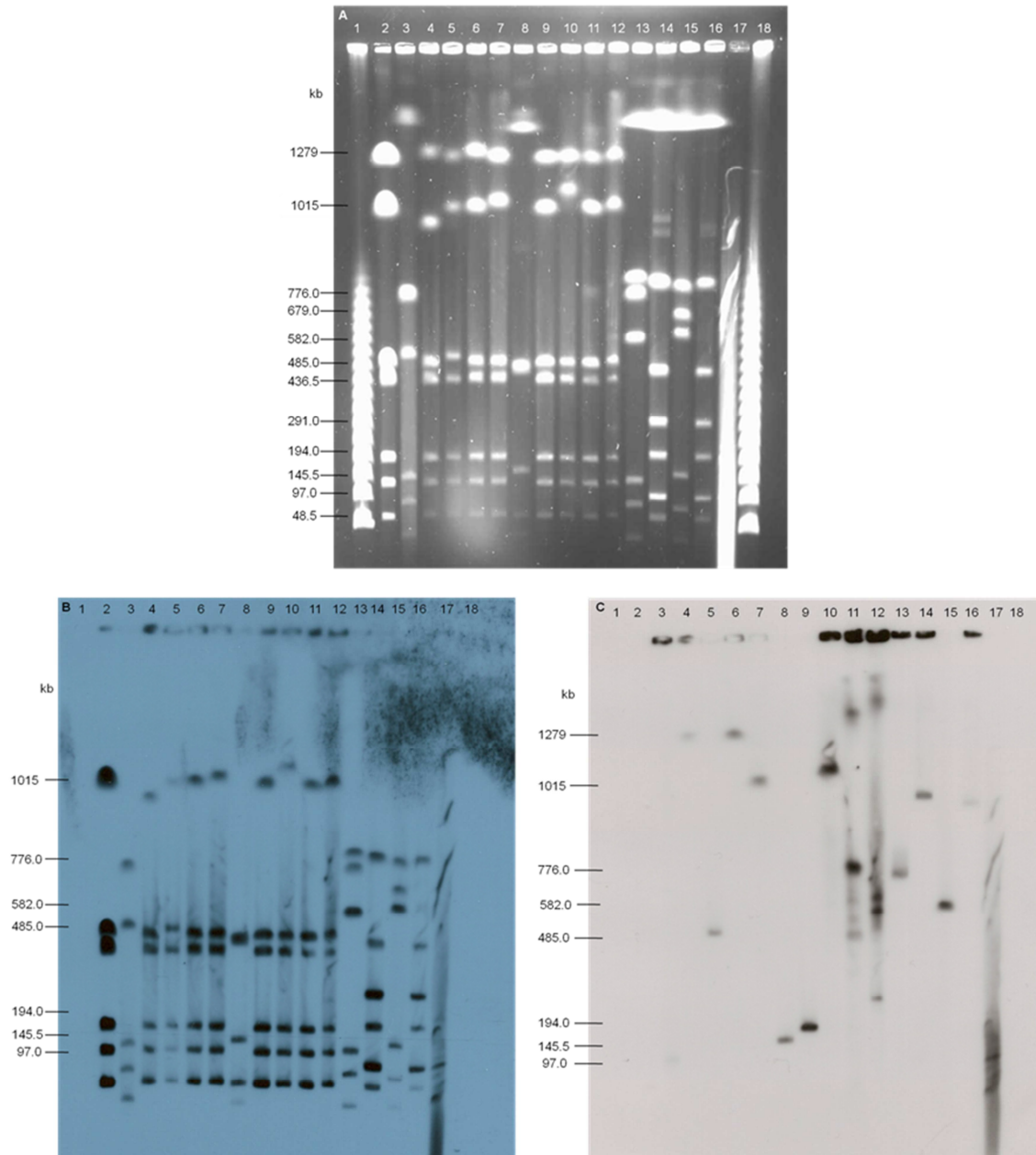


Figure 6. Pulse-field gel electrophoresis (PFGE) and Southern blot hybridization. A) PFGE of I-CeuI-digested total DNA of recipient, donor and transformant bacteria; B) corresponding Southern hybridization with 16S rDNA probe; C) corresponding Southern hybridization with *int11* probe. Lane 1 – lambda PFG marker (New England Biolabs); 2 – *A. baylyi* BD413 (recipient); 3 – *S. enterica* serovar Typhimurium 490 (donor); 4-7 – transformants from exposure to DNA of *S. enterica* serovar Typhimurium 490, (St)1, SD1, (St)2, (St)3, respectively; 8 – *A. baumannii* 064 (donor); 9-12 – transformants from exposure to DNA of *A. baumannii* 064, SD2, (AbII)1, (AbII)2 and (AbII)3, respectively; 13 – *S. enterica* serovar Rissen 486 (donor); 14 – *E. cloacae* C2R371 (donor); 15 – *E. coli* C10R379 (donor); 16 – *C. freundii* C16R385 (donor); 17 – *E. fergusonii* AS041A2 (donor); 18 – lambda PFG marker (New England Biolabs).

4.2 DNA sequencing

Sequencing of the flanking regions of the class 1 integrons carried by the donor bacteria revealed that their insertion sites varied, and that the integrons were often linked to transposable elements.

A. baumannii 064

The class 1 integron of *A. baumannii* 064 was found to be inserted in a Tn1721-like transposon (Schmitt et al., 1979; Allmeier et al., 1992), which contains the transposition genes *tnpA* (74 % similar to the *tnpA* gene of Tn1721) and *tnpR*, the tetracycline resistance gene *tetA* and its repressor *tetR*. This transposon is flanked by the insertion sequence IS26 on both sides, forming an IS26-composite transposon. On the 5'-CS side of the integron, a few chromosomal genes are located between the IS26 and the Tn1721-like genes (see Figure 9). The class 1 integron, the Tn1721-like and the chromosomal genes seem to have been inserted in its current genomic position by transposition, as they are flanked by an interrupted transposase and a TSD of 12 bp (CGACCGAAATGC). On the 3'-CS side, more chromosomal genes were found after the IS26 (see Figure 9). In total, about 13 kbs and 6.8 kbs were sequenced flanking the 5'-CS and the 3'-CS of the class 1 integron, respectively.

A. baumannii 65FFC

In *A. baumannii* 65FFC, a total of 3258 bp of sequence was determined adjacent to the 5'-CS region and 3564 bp next to the 3'-CS region. The integron In76 was found embedded in a Tn402-like transposon. A MITE-like structure of 439 bp was identified immediately adjacent to the beginning of the 5'-CS of the integron, preceded by an incomplete putative transposase. Downstream of the 3'-CS, the two genes (*tniBΔ1* and *tniA*) that were identified belong to a common defective transposition module of integrons. However, the *tniA* gene was interrupted by a second MITE with an identical 439 bp sequence to the one at the 5'-CS flanking region. An interrupted putative transposase also followed the

MITE structure located after the 3'-CS. A 5 bp TSD (TCCAT) was identified on each side of the MITE (Figure 7B). The fact that the host gene was interrupted by the single insertion of the MITE-integron-MITE, together with the TSD, strongly suggested that the entire structure had inserted into the transposase gene through transposition promoted by the MITEs. The nucleotide sequence from the flanking region as well as the conserved and variable regions of the class 1 integron obtained was deposited in the GenBank database with the accession number JF810083.

Bioinformatics also revealed that the 439 bp MITE identified in the Portuguese *A. baumannii* strain was 100 % identical with a previously identified MITE flanking the class 1 integron of the *A. johnsonii* NFM2, isolated from the digestive tract of an ocean prawn in Australia (GenBank accession number FJ711439). A 5 bp TSD (GTTGC) was also identified surrounding the latter MITEs, and the acquisition of the integron by *A. johnsonii* by a MITE-facilitated transposition-like mechanism was also proposed by Gillings and collaborators (Gillings et al., 2009a). Although the MITE-like structures are inserted in the same relative position in relation to the integron, they flank two different class 1 integrons with unrelated gene cassettes, and the entire structures are inserted into a different genomic context (Figure 7B and C).

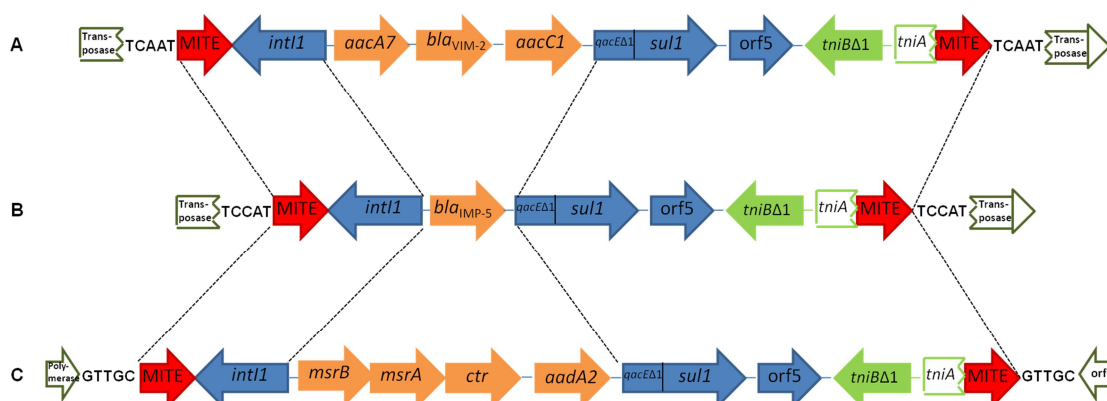


Figure 7. Schematic representation of the flanking regions of class 1 integrons. A – integron of *A. bereziniae* 118FFC (GenBank JX235356); B – integron of *A. baumannii* 65FFC (GenBank JF810083); C – integron of *A. johnsonii* NFM2 (GenBank FJ711439). The regions between the dashed lines are 100 % identical (1720/1720 bp and 3728/3728 bp).

P. aeruginosa SM

In *P. aeruginosa* SM, 1002 bp were sequenced and the *tnpR* and *tnpM* genes were identified adjacent to the 5'-CS flanking region of the integron; the *tnp* genes were 100 % identical to the genes found in the Tn5051-like transposon, indicating that the integron is probably located in a transposon. The sequence obtained from the flanking region of the 3'-CS always showed low quality, and the genes flanking this region of the integron could not be determined. A possible cause is that the integron was inserted in a region that it is present in more than one copy in the bacterial chromosome.

S. enterica serovar Rissen 486

In *S. enterica* serovar Rissen 486, the 4354 bp sequence obtained revealed the *tnpA*, *tnpR* and *tnpM* genes next to the 5'-CS region of the integron. The closest homologues to the *tnp* genes were found in the transposon Tn1721, suggesting also that the class 1 integron in serovar Rissen 486 may be located inside a transposon. Part of a *chrA* gene, which codes for a putative truncated chromate ion transporter, was found in the 739 bp sequence obtained flanking the 3'-CS region. We were unable to obtain additional DNA sequence by primer walking. Probably, also in this case, the integron was inserted in a region that it is present in more than one copy in the genome of the isolate.

S. enterica serovar Typhimurium 490

In *S. enterica* serovar Typhimurium 490, the integron was inserted in an intact Tn21-like transposon, containing all the genes that are part of this transposon, with the only difference being the gene cassette array present in the variable region of the class 1 integron (Liebert et al., 1999). This includes the 3 genes involved in transposition, *tnpA*, *tnpR* and *tnpM*, adjacent to the 5'-CS, and the ISs, *tni* and *urf2* genes and the *mer* operon on the 3'-CS flanking region (11 500 bp). Adjacent to the 5'-CS, 6632 bp were determined and, in addition to the Tn21 transposition genes, the *ybjA*, *catA1* and IS1 genes were identified (see Figure 14).

The sequence of the flanking regions of the class 1 integrons of the other donor bacteria used, namely *C. freundii* C16R385, *E. cloacae* C2R371, *E. coli* C10R379 and *E. fergusonii* AS041A2, was not determined.

5. Detection of MITEs

The detection of a MITE-like structure with 439 bp flanking the class 1 integron of *A. baumannii* 65FFC lead us to screen the presence of this MGE in our *Acinetobacter* spp. isolates, including 28 clinical isolates. Only the *A. bereziniae* 118FFC was positive for the MITEs; the sequence of the PCR-product revealed a 100 % identical nucleotide composition with the MITEs present in *A. baumannii* 65FFC and *A. johnsonii* NFM2. PCRs done to determine the relative position of the MITE to the integron showed that the MITEs were exactly in the same position in *A. bereziniae* 118FFC as in *A. baumannii* 65FFC. The sequencing of these PCR-products, the variable region and the conserved regions of the class 1 integron unravelled the genomic context of the integron present in *A. bereziniae* 118FFC that is surrounded by two MITE-like structures. In addition, a TSD with 5 bp (TCAAT) was detected on each side of the MITEs, suggesting that the structure composed by the MITE-integron-MITE was acquired by transposition. This mode of acquisition was reinforced by the fact that a single gene (*tnpA*) was interrupted by the insertion of the MITE-integron-MITE (Figure 7A). The nucleotide sequence obtained from *A. bereziniae* 118FFC was deposited in the GenBank database, with the accession number JX235356.

The three class 1 integrons surrounded by the 439 bp MITEs for which the conserved and flanking regions are sequenced only differ from each other in the variable region of the integron and in the genes where the entire structure was inserted (Figure 7). In *A. baumannii* 65FFC and *A. bereziniae* 118FFC the insertion site was a transposase gene.

In silico analysis of the MITEs sequence revealed a partial alignment with class 1 integron 5'-CS flanking region from several clinical *Acinetobacter* spp. (strains

694, 695, 696, 5227, 5248 and 9043) isolates collected in Brazil (Mendes et al., 2007). PCR detection of the 439 bp MITE was positive for these isolates, which include six *A. baumannii*. The sequence of the PCR-product confirmed a 100 % identity of the 439 bp MITEs. The same relative position of the MITEs in relation to the integrons present in the other MITEs-containing isolates was confirmed by PCR. Therefore, we can assume that the genetic context of all the class 1 integrons where the 439 bp MITEs were detected is the same.

6. Antimicrobial susceptibility

The antimicrobial susceptibility profile of the integron-carrying bacteria was mostly determined for the antibiotic resistance expected from the presence of the gene cassettes in the integron, but in some cases also for additional susceptibilities. Determination of the MIC for the antibiotics encoded by the gene cassettes in donor and recipient bacteria allowed the determination of the concentration used to select transformants obtained in the natural transformation assays, and also to record changes in the antimicrobial susceptibility profile of the transformants. Susceptibility testing to other antimicrobials, not encoded in the gene cassettes of the integrons, allowed us to detect acquisition of additional genes or traits by transformed cells. The MICs of the donor bacteria *E. coli* K71-77, *K. pneumoniae* K66-45 and *P. aeruginosa* K34-73, which were only used as donor DNA to screen transformants with phenotypic changes, were also determined; some of the MICs have been previously determined (Samuelsen et al., 2010; Samuelsen et al., 2011).

The MICs of the donor and recipient bacteria to different antibiotics are shown in Table 7. *A. baylyi* BD413 is resistant to trimethoprim and rifampicin, with the MIC > 32 µg/ml for both antibiotics. Therefore, MICs for these antibiotics were not determined in the donors that carry a gene cassette related with such resistance.

Table 7. Minimal Inhibitory Concentration (MIC; µg/ml) of wild-type recipient and donor bacteria as determined by the Etest method.

Bacteria	AM	CAZ	CN	CTX	K	SC	SX	TOB
<i>A. baylyi</i> BD413	1.0	1.5	0.064	1.5	0.38	3	16	0.5
<i>A. baumannii</i> 064	64	> 256	n.d.	> 256	> 256	n.d.	> 1024	n.d.
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	n.d.	> 1024	n.d.
<i>C. freundii</i> C16R385	n.d.	n.d.	n.d.	n.d.	n.d.	192	> 1024	n.d.
<i>E. cloacae</i> C2R371	n.d.	n.d.	n.d.	n.d.	n.d.	192	> 1024	n.d.
<i>E. coli</i> C10R379	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	> 1024	n.d.
<i>E. coli</i> K71-77	> 256	> 256	> 1024	> 256	n.d.	n.d.	> 1024	> 256
<i>E. fergusonii</i> AS041A2	n.d.	n.d.	n.d.	n.d.	n.d.	8	> 1024	n.d.
<i>K. pneumoniae</i> K66-45	> 256	> 256	> 1024	> 256	n.d.	n.d.	> 1024	> 256
<i>P. aeruginosa</i> K34-73	n.d.	> 256	> 256	n.d.	n.d.	n.d.	> 1024	> 256
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024	> 1024	n.d.
<i>S. enterica</i> Rissen 486	> 256	n.d.	n.d.	n.d.	n.d.	> 1024	> 1024	n.d.
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	192	> 1024	n.d.

AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole; TOB – tobramycin.
n.d. – not determined.

II. Natural transformation

The different natural transformation outcomes were evaluated in terms of transformation frequency and phenotypic and genotypic characterization of the obtained transformants.

The transformants obtained were named based on the code “[recipient](donor)isolate number”. Whenever the recipient is not mentioned, the *A. baylyi* BD413 strain was used. The designation of each donor was done with an abbreviation: Abl – *A. baumannii* 65FFC; Abll – *A. baumannii* 064; Cf – *C. freundii* C16R385; Eclo – *E. cloacae* C2R371; Ecl – *E. coli* C10R379; Ecll – *Escherichia coli* K71-77; Ef – *E. fergusonii* AS041A2; Kp – *Klebsiella pneumoniae* K66-45; Ps – *P. aeruginosa* SM; Sr – *S. enterica* serovar Rissen 486; St – *S. enterica* serovar Typhimurium 490. For convenience, six of the *A. baylyi* transformants that were used in different experiments were named from

SD1 to SD6. The transformants SD1 and SD2 were obtained in transformation of *A. baylyi* BD413 by DNA from *S. enterica* serovar Typhimurium 490 and *A. baumannii* 064, respectively. Transformants SD3, SD4, SD5 and SD6 were obtained in transformation of *A. baylyi* SD2 by DNA from *A. baumannii* 65FFC, *S. enterica* serovar Typhimurium 490, *S. enterica* serovar Rissen 486 and *P. aeruginosa* SM, respectively.

Several transformants were obtained in the different transformation assays and further analysed. However, only a few transformants, which represent the single isolates obtained, are described in the below sections.

1. Interspecies transfer of class 1 integrons into wild-type *A. baylyi*

Natural transformation of *A. baylyi* BD413 was done with nine different class 1 integron-carrying wild-type bacteria, representing seven different species.

1.1 Transformation frequency

Natural transformation of *A. baylyi* BD413 by class 1 integron-containing DNA from the related species *A. baumannii* occurred at frequencies up to 1.6×10^{-7} transformants per recipient over a 24 hrs period. Natural transformation was also seen after exposure to integron-containing DNA of strains from the unrelated bacterial species *C. freundii*, *E. cloacae*, *P. aeruginosa*, *S. enterica* serovar Rissen and *S. enterica* serovar Typhimurium, at frequencies ranging from 1.9×10^{-8} to 1.8×10^{-7} (Table 8). The only two integron-containing DNA sources tested and not capable of transforming wild-type *A. baylyi* BD413 above the detection limit of 1 transformant per 10^9 bacteria were two *Escherichia* spp. isolates (*E. coli* C10R379 and *E. fergusonii* AS041A2). For *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490, the transformation frequency was also calculated as transformants per exposed unit of DNA; it was

1.3×10^{-8} and 7.5×10^{-8} transformants per genome equivalent, respectively. In addition to purified DNA, the supernatant of a heat-killed bacterial cell suspension also gave rise to bacterial transformants (Table 8) at comparable frequencies. Thus, DNA purity seems to be of limited importance in natural transformation of *A. baylyi*.

The transformation frequency obtained with DNA from positive control source (*A. baylyi* KTG with the *ntpII* gene) was 4.7×10^{-4} . The efficiency of the control transformation showed that the recipient cells achieved the competent state during the natural transformation assays and successfully took up and integrated the incoming DNA. Transformation without DNA (negative control) did not yield transformants (data not shown).

Table 8. Natural transformation of *A. baylyi* BD413 with class 1 integron-containing total DNA from different sources.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients ^a
<i>A. baylyi</i> BD413	<u>Purified DNA:</u>			
	<i>A. baumannii</i> 064	$(3.2 \pm 3.1) \times 10^1$	$> (1.5 \pm 0.4) \times 10^9$	$< 2.1 \times 10^{-8b}$
	<i>A. baumannii</i> 65FFC	$(2.6 \pm 2.5) \times 10^2$	$> (1.6 \pm 0.4) \times 10^9$	$< 1.6 \times 10^{-7}$
	<i>C. freundii</i> C16R385	$(5.1 \pm 4.0) \times 10^0$	$(4.3 \pm 0.8) \times 10^8$	1.2×10^{-8}
	<i>E. cloacae</i> C2R371	$(5.1 \pm 2.3) \times 10^0$	$(2.8 \pm 0.0) \times 10^8$	1.9×10^{-8}
	<i>E. coli</i> C10R379	0	$(7.9 \pm 4.4) \times 10^8$	0
	<i>E. fergusonii</i> AS041A2	0	$> 1.7 \times 10^9$	0
	<i>P. aeruginosa</i> SM	$(4.4 \pm 4.3) \times 10^1$	$> (1.6 \pm 0.5) \times 10^9$	$< 2.8 \times 10^{-8}$
	<i>S. enterica</i> Rissen 486	$(3.1 \pm 3.0) \times 10^2$	$> (1.7 \pm 0.2) \times 10^9$	$< 1.8 \times 10^{-7}$
	<i>S. enterica</i> Typhimurium 490	$(1.4 \pm 1.3) \times 10^2$	$> (1.4 \pm 0.6) \times 10^9$	$< 1 \times 10^{-7b}$
	<u>Supernatant/lysate with DNA:</u>			
	<i>A. baumannii</i> 064	$(1.7 \pm 0.1) \times 10^2$	$> (1.9 \pm 0.4) \times 10^9$	$< 8.9 \times 10^{-8}$
	<i>A. baumannii</i> 65FFC	$(2.0 \pm 1.5) \times 10^2$	$> (1.7 \pm 0.0) \times 10^9$	$< 1.2 \times 10^{-7}$
	<i>P. aeruginosa</i> SM	$(1.7 \pm 0.7) \times 10^2$	$> (1.2 \pm 0.7) \times 10^9$	$< 1.4 \times 10^{-7}$
	<i>S. enterica</i> Rissen 486	$(3.6 \pm 0.2) \times 10^2$	$> (1.7 \pm 0.0) \times 10^9$	$< 2.1 \times 10^{-7}$
	<i>S. enterica</i> Typhimurium 490	$(3.6 \pm 0.9) \times 10^1$	$(8.1 \pm 5.2) \times 10^9$	4.4×10^{-8}

^a The transformation frequency obtained with DNA from positive control source (*A. baylyi* KTG with the *ntpII* gene) was 4.7×10^{-4} .

^b The transformation frequency is, when calculated as the frequency per exposed unit of DNA: 1.3×10^{-8} transformants per genome equivalent and 7.5×10^{-8} transformants per genome equivalent for *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490, respectively.

1.2 Phenotypic characterization of *A. baylyi* transformants

Some transformants were randomly selected for detailed analysis. First, antimicrobial susceptibility profiles were determined. When the antimicrobial susceptibility profile of the transformants detected by the disc diffusion method was altered in relation with the recipient, the Etest method was used to assess the MICs (Table 9). Transformants obtained in transformations with DNA from *C. freundii* C16R385 and *E. cloacae* C2R371 did not show relevant changes and therefore MICs are not shown. Some of the transformants showed resistance to the antibiotics used in selection and had a susceptibility profile that could be explained by the acquisition of the class 1 integron of the donor. However, the susceptibility profiles of other transformants were altered in ways that could not be explained by the resistance phenotypes encoded by the transferred complete integron. Many of these phenotypic transformants mainly showed reduced susceptibility to ampicillin and sulphonamides, when compared with the profile of the unexposed recipient bacterium.

Table 9. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baylyi</i> BD413	1.0	1.5	0.064	1.5	0.38	3	16
<i>A. baumannii</i> 064	> 256	n.d.	> 256	n.d.	> 256	n.d.	> 1024
<i>A. baylyi</i> SD2	0.75	n.d.	16	n.d.	96	n.d.	> 1024
(AbII)1	0.75	n.d.	48	n.d.	192	n.d.	> 1024
(AbII)2	1.0	n.d.	32	n.d.	> 256	n.d.	> 1024
(AbII)3	1.0	n.d.	64	n.d.	> 256	n.d.	> 1024
(AbII)4	48	n.d.	0.38	n.d.	0.75	n.d.	16
(AbII)5	12	n.d.	0.5	n.d.	1.0	n.d.	32
(AbII)6	1.0	n.d.	0.38	n.d.	> 256	n.d.	48
(AbII)7	96	n.d.	0.125	n.d.	0.75	n.d.	64
(AbII)8	16	n.d.	0.19	n.d.	1.0	n.d.	94
(AbII)L1	32	n.d.	0.38	n.d.	0.5	n.d.	24
(AbII)L2	4	n.d.	0.19	n.d.	0.5	n.d.	48

(continue)

Table 9 (continued)

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	n.d.	> 1024
<i>A. baylyi</i> (Abl)L1	6	8	n.d.	8	n.d.	n.d.	16
(Abl)L2	4	8	n.d.	8	n.d.	n.d.	64
(Abl)L3	0.75	1.5	n.d.	1.5	n.d.	n.d.	24
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> (Ps)1	6	n.d.	0.19	n.d.	n.d.	3	24
(Ps)L1	6	n.d.	0.5	n.d.	n.d.	4	24
(Ps)L2	6	n.d.	0.38	n.d.	n.d.	3	24
(Ps)L3	4	n.d.	n.d.	n.d.	n.d.	3	32
<i>S. enterica</i> Rissen 486	> 256	n.d.	n.d.	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> (Sr)1	4	n.d.	n.d.	n.d.	n.d.	3	16
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> (St)1	32	n.d.	n.d.	n.d.	n.d.	12	> 1024
SD1	6	n.d.	n.d.	n.d.	n.d.	12	> 1024
(St)2	3	n.d.	n.d.	n.d.	n.d.	8	> 1024
(St)3	12	n.d.	n.d.	n.d.	n.d.	32	> 1024
(St)L1	6	n.d.	n.d.	n.d.	n.d.	3	64
(St)L2	6	n.d.	n.d.	n.d.	n.d.	3	32

^a For each transformant, the name is given by the code (donor)isolate number. The *A. baylyi* BD413 strain was the recipient. Abl – *Acinetobacter baumannii* 65FFC; AblI – *Acinetobacter baumannii* 064; Ps – *Pseudomonas aeruginosa* SM; Sr – *Salmonella enterica* serovar Rissen 486; St – *Salmonella enterica* serovar Typhimurium 490. When the isolate number is preceded by an “L”, transformants were obtained with lysate DNA as donor. Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole. n.d. – not determined.

1.3 Genotypic characterization of *A. baylyi* transformants

1.3.1 Class 1 integron targeting PCR

Transformants with altered antimicrobial susceptibility profiles were further analysed to detect acquired resistance genes. Among all the phenotypes with increased resistance observed after exposure of the BD413 strain to DNA extracted from the various integron-carrying species, four transformants were

shown to have acquired the complete class 1 integron from *S. enterica* serovar Typhimurium 490 [named SD1, (St)1, (St)2 and (St)3], and another four transformants the entire class 1 integron from *A. baumannii* 064 [named SD2, (AbII)1, (AbII)2 and (AbII)3], as confirmed by class 1 integron specific PCR (Figure 8) and DNA sequencing.

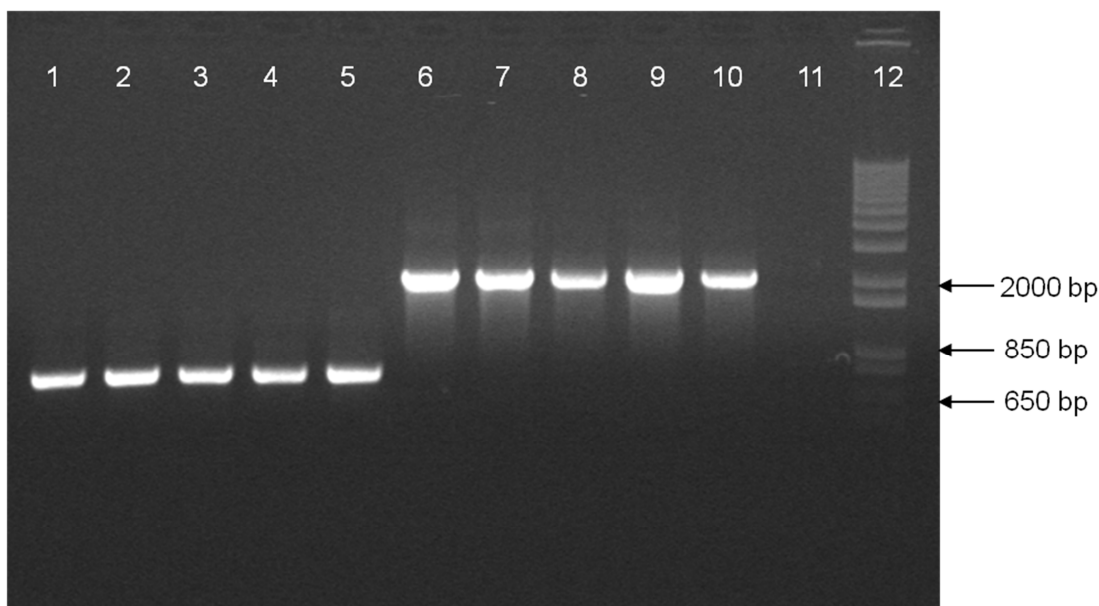


Figure 8. Agarose gel electrophoresis of class 1 integron PCR-products. Lane 1 – *A. baumannii* 064 (donor strain); 2-5 – transformants from *A. baumannii* 064, SD2, (AbII)1, (AbII)2 and (AbII)3, respectively; 6 – *S. enterica* serovar Typhimurium 490 (donor strain); 7-10 – transformants from *S. enterica* serovar Typhimurium 490, SD1, (St)1, (St)2 and (St)3, respectively; 11 – *A. baylyi* BD413 (recipient strain); 12 – 1 Kb Plus DNA ladder (Invitrogen).

In all the eight transformants that acquired the complete class 1 integron, flanking regions were determined; additional flanking DNA was acquired by the transformants. Flanking acquired DNA fragments were up to 23 000 bp long and included in several cases transposable elements or other genes with important phenotypes, such as a chloramphenicol resistance gene (*catA2*). Detailed description of the regions acquired by these transformants is given below.

1.3.2 PFGE and Southern blot hybridization

The possible transfer of whole plasmids was investigated in the transformants collected after exposure to DNA from the plasmid-harboring *S. enterica* serovar Typhimurium 490 or *A. baumannii* 064. Although plasmids were clearly identified in both of the donor species by agarose gel-electrophoresis or PFGE, the same plasmids could not be detected in the transformants.

The *A. baylyi* transformants SD1, (St)3, SD2 and (AbII)1 were shown, by PFGE and probe hybridization, to carry the acquired class 1 integron in the chromosome (Figure 6, lanes 5, 7, 9 and 10, respectively).

Co-hybridization with the two probes targeting the *intl1* and the 16S rDNA genes did not take place for DNA extracted from transformants (St)1 and (St)2. Chromosomal location is nevertheless assumed because the larger fragment, where the integron of these two transformants is located, is not expected to hybridize with the 16S rDNA probe even though it is chromosomal. This is due to the opposite orientation of the rRNA operons at nucleotides 1660700-1666022 and 2941950-2947363 (GenBank accession number CR543861), leading to a 1.28 Mb I-Ceul fragment without the 16S rDNA gene. The seven rRNA operon fragments of *A. baylyi* can be seen for strain BD413 and transformants (St)1 and (St)2 (Figure 6A, lanes 2, 4 and 6, respectively), as well as hybridization of the *intl1* probe in the bigger fragment of transformants (St)1 and (St)2 (Figure 6C, lanes 4 and 6, respectively).

Variable locations, as well as different numbers, of the hybridizing band in the PFGE results of transformants (AbII)2 and (AbII)3 (Figure 6, lanes 11 and 12, respectively) led to several repetitions of the experiments; however both transformants repeatedly displayed a variable chromosomal location of the integrase sequence (data not shown). The chromosomal location of the integron was also confirmed by full genome sequence of one of these transformants, the (AbII)3. This variability may hypothetically be due to gene amplification, which is reported to occur in *A. baylyi* (Reams and Neidle, 2003, 2004), followed by intragenomic rearrangements. Rearrangements are often associated with repetitive sequences (Hacker et al., 2003), which are known to represent 1.6 %

of the *A. baylyi* genome, including short repeats, ISs, Tns, clusters encoding transposases and prophages (Barbe et al., 2004).

1.3.3 DNA sequencing

Transformants obtained from the A. baumannii 064 donor DNA

The *A. baylyi* transformant SD2, obtained after exposure to *A. baumannii* 064 DNA, acquired considerable stretches of the genomic donor DNA flanking the complete integron (Figure 9). Extensive primer walking identified a crossover in the *lrp* gene of both the donor and recipient genomes, 5428 bp from the 3'-CS region of the integron. The second crossover junction was not identified but, based on primer walking results, had to be located at least at a distance of 10 500 bp from the 5'-CS region of the integron, which was the length of the sequence determined on this side. As in the donor, the acquired DNA includes genes similar to the Tn1721 transposon. The high nucleotide similarities between the *lrp* gene of the donor and the recipient and the characteristics of the crossover junctions suggest that *recA*-dependent homologous (also called homeologous, heterologous, or heterogamic) recombination had facilitated the acquisition of the integron-containing DNA (Figure 10), a process recently investigated in detail for *A. baumannii* donor and *A. baylyi* recipient cells (Ray et al., 2009).

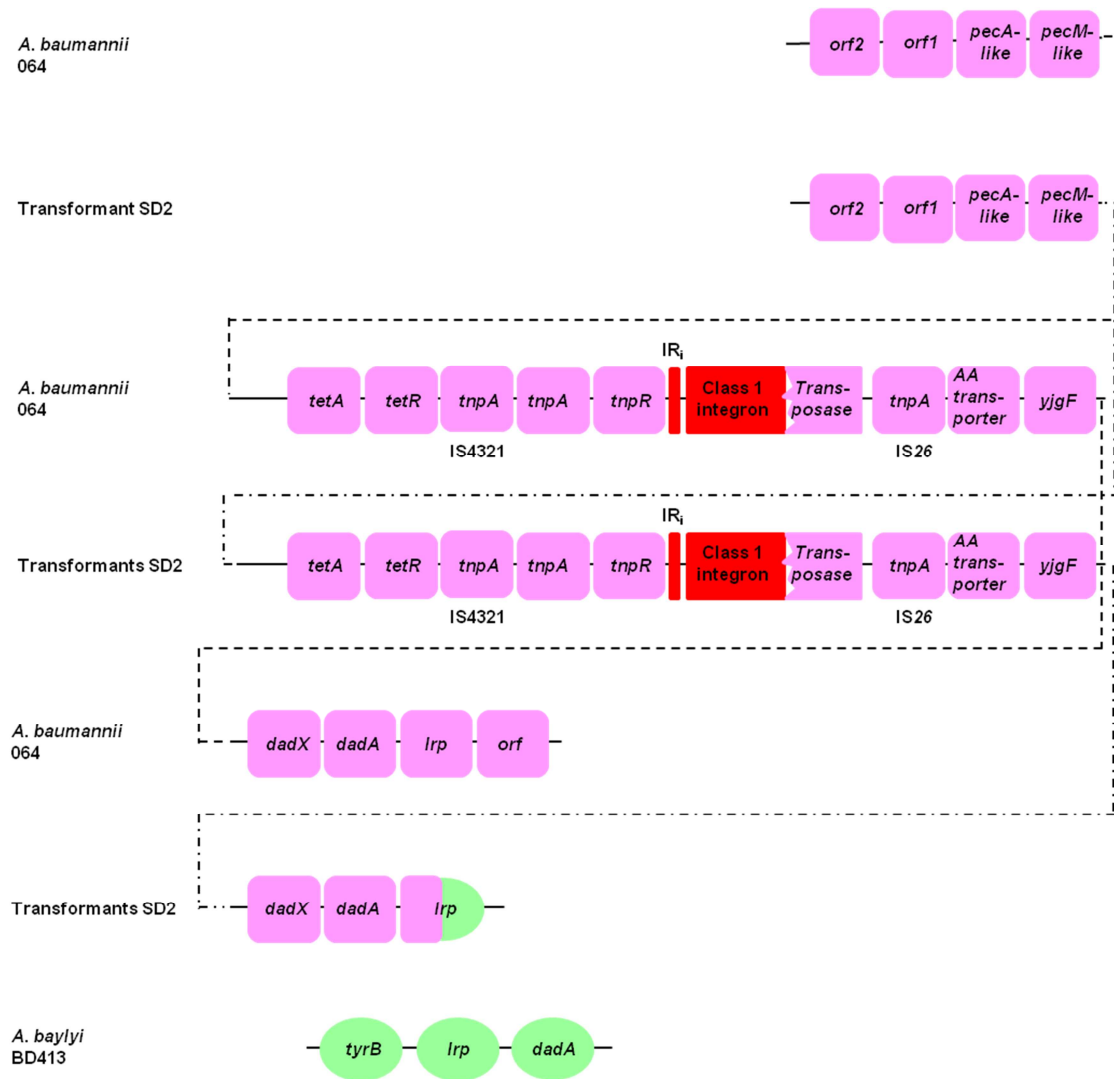


Figure 9. DNA fragment acquired by transformant *A. baylyi* SD2 from *A. baumannii* 064. The class 1 integron includes the *intI1*, one gene cassette, and the *qacΔE*, *sull* and *orf5* genes.

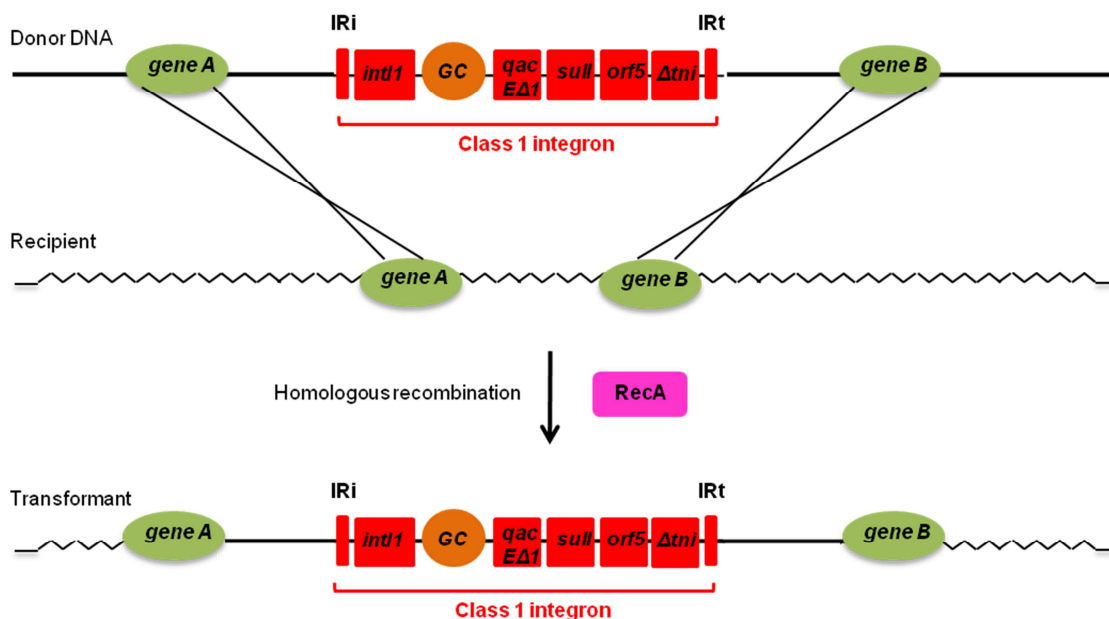


Figure 10. Schematic representation of the horizontal acquisition of a class 1 integron by homologous recombination.

Whole genome sequencing of the *A. baylyi* transformant (AbII)3 revealed that it had acquired 18 625 bp of DNA sequence from the *A. baumannii* 064 genome, which contained the IS26-composite transposon (Figure 11); the horizontally acquired fragment was submitted to GenBank under the accession number JX041889. The inserted DNA contained the integron embedded in the Tn1721-like region, further flanked on both sides by the IS26 elements. A TSD of 8 bp (ACTCGATG) on both sides of the IS26 insertion suggests that transfer of the entire 18 625 bp segment occurred by transposition facilitated by the IS26 sequences (Figure 12), into the ACIAD0480 gene of *A. baylyi* (encoding a putative membrane protein).

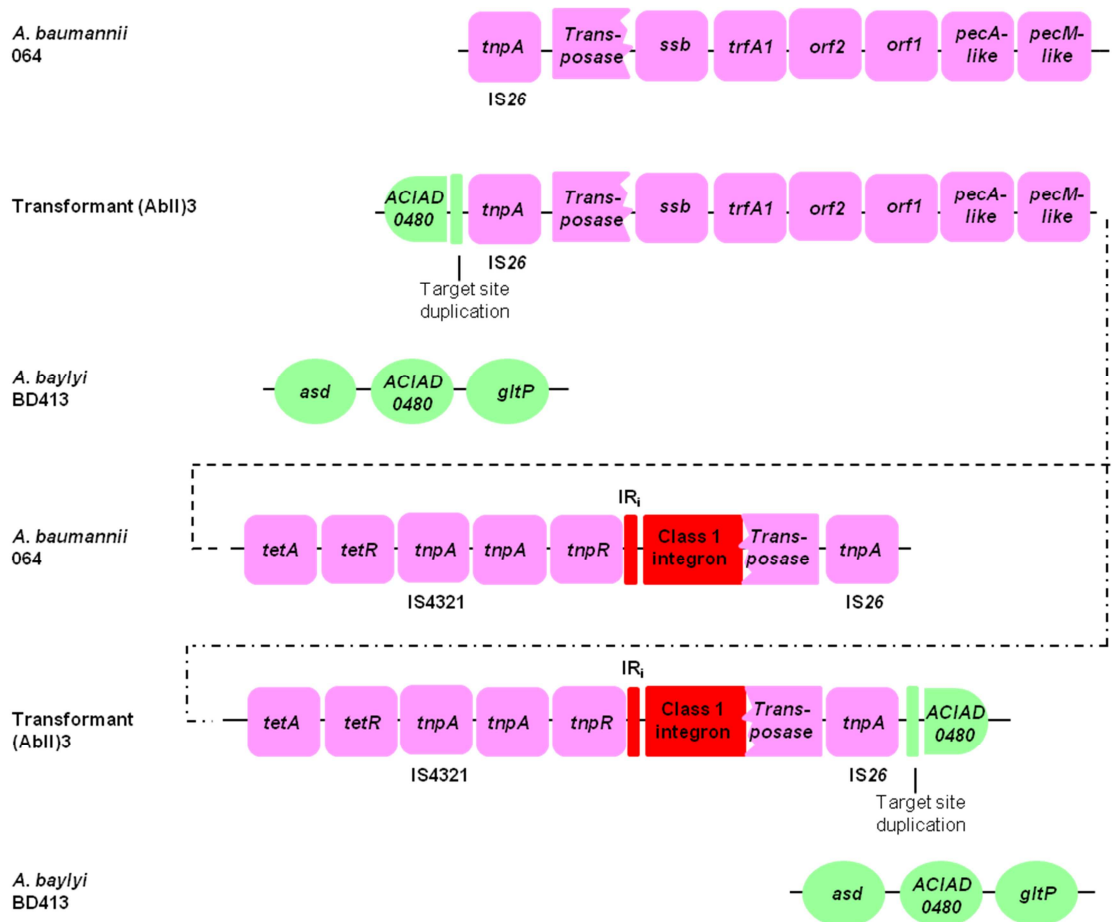


Figure 11. DNA fragment acquired by transformant *A. baylyi* (AbII)3 from *A. baumannii* 064. The class 1 integron includes the *intI1*, one gene cassette, and the *qacΔE*, *sull* and *orf5* genes.

Analysis of the additional transformants obtained from DNA of *A. baumannii* 064 by primer walking allied with PCR confirmed that the integrons acquired by transformants (AbII)1 and (AbII)2 were also located within the Tn1721-like transposon, and flanked by the IS26 element. However, further sequence could not be determined because of the presence of two copies of the IS26, which did not allowed primer walking after this region. Therefore, the exact location of the acquired DNA in the *A. baylyi* genome could not be determined, but the obtained DNA sequence suggests a similar composition and mode of transfer as for transformant (AbII)3.

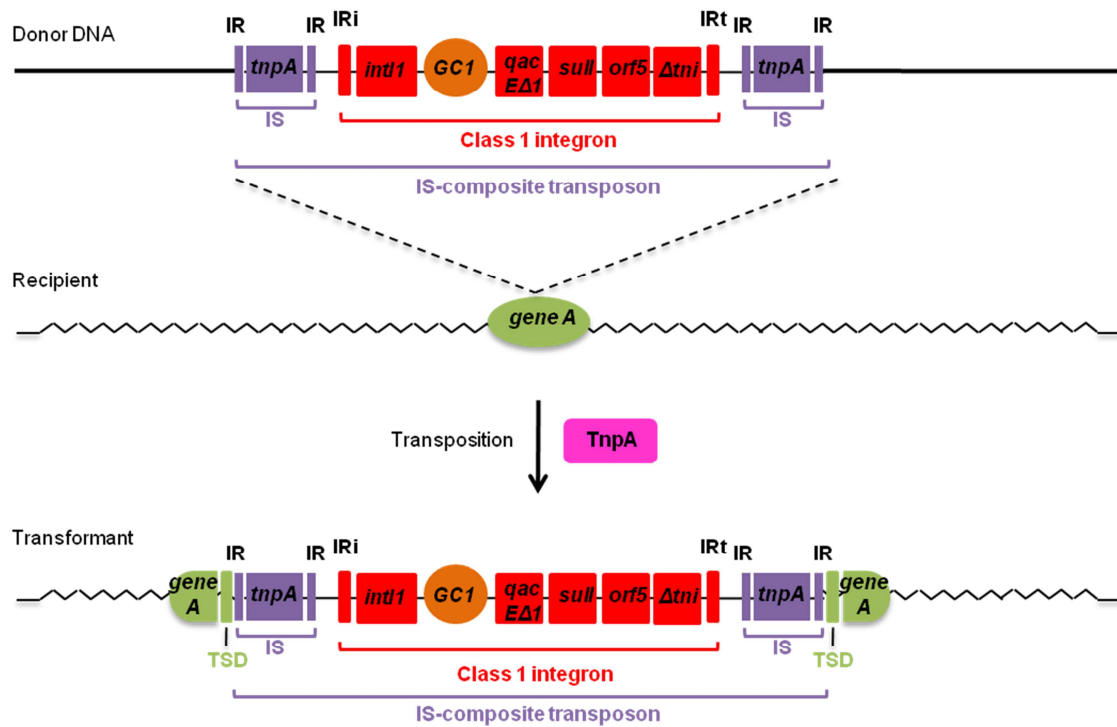


Figure 12. Schematic representation of the horizontal acquisition of an IS-composite transposon by transposition, with duplication of sequences around the insertion site (TSD).

Transformants obtained from the S. enterica serovar Typhimurium 490 donor DNA

In the transformants (St)1 and SD1, obtained after exposure to DNA of *S. enterica* serovar Typhimurium 490, the incorporated DNA region was found to consist of a copy of the Tn21-like transposon, which also contained the class 1 integron; around 20 500 bp were acquired. In both cases, TSDs of 4 (TTTA) and 5 (TTAAG) bp, respectively, were identified flanking the acquired transposon, providing evidence that the (St)1 and SD1 transformants resulted from active transposition of the Tn21-like (Figure 13). Additionally, the transformant SD1 showed a partial duplication of the *merR* gene of the transposon. In transformant (St)1, the integron-containing transposon was inserted into the *A. baylyi* gene ACIAD1773, which encodes a putative transport protein permease (Figure 14). In transformant SD1, the integron-carrying

transposon was inserted into the gene ACIAD3230, encoding a putative proton/sodium-glutamate symport protein (Figure 15).

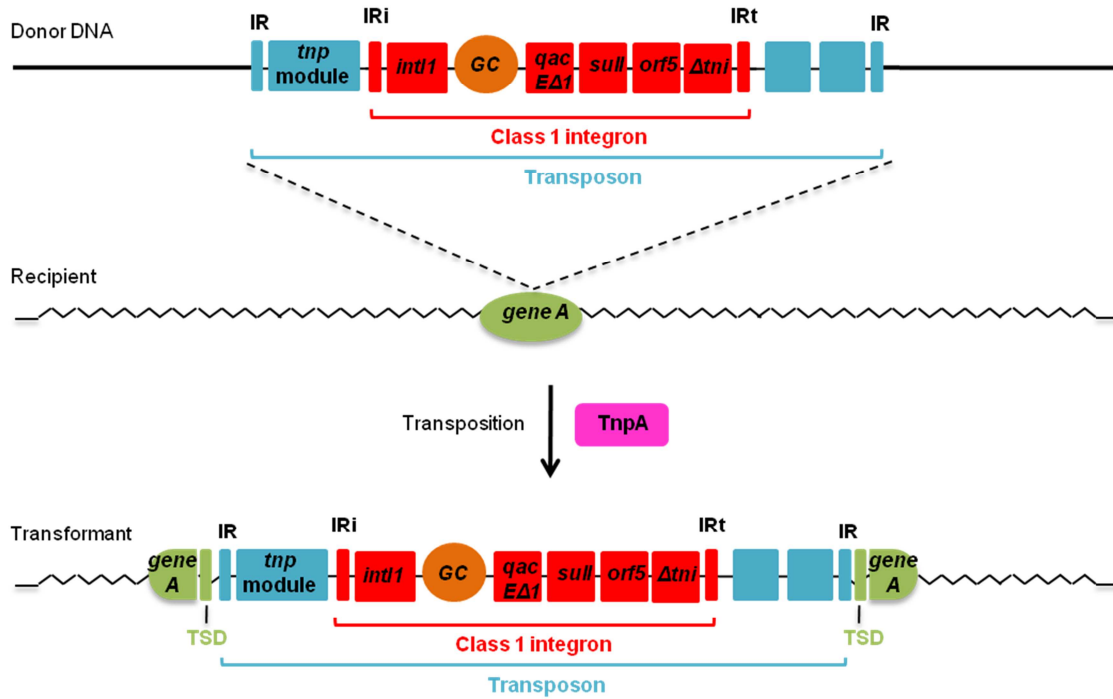


Figure 13. Schematic representation of the horizontal acquisition of a transposon by transposition, with duplication of sequences around the insertion site (TSD).

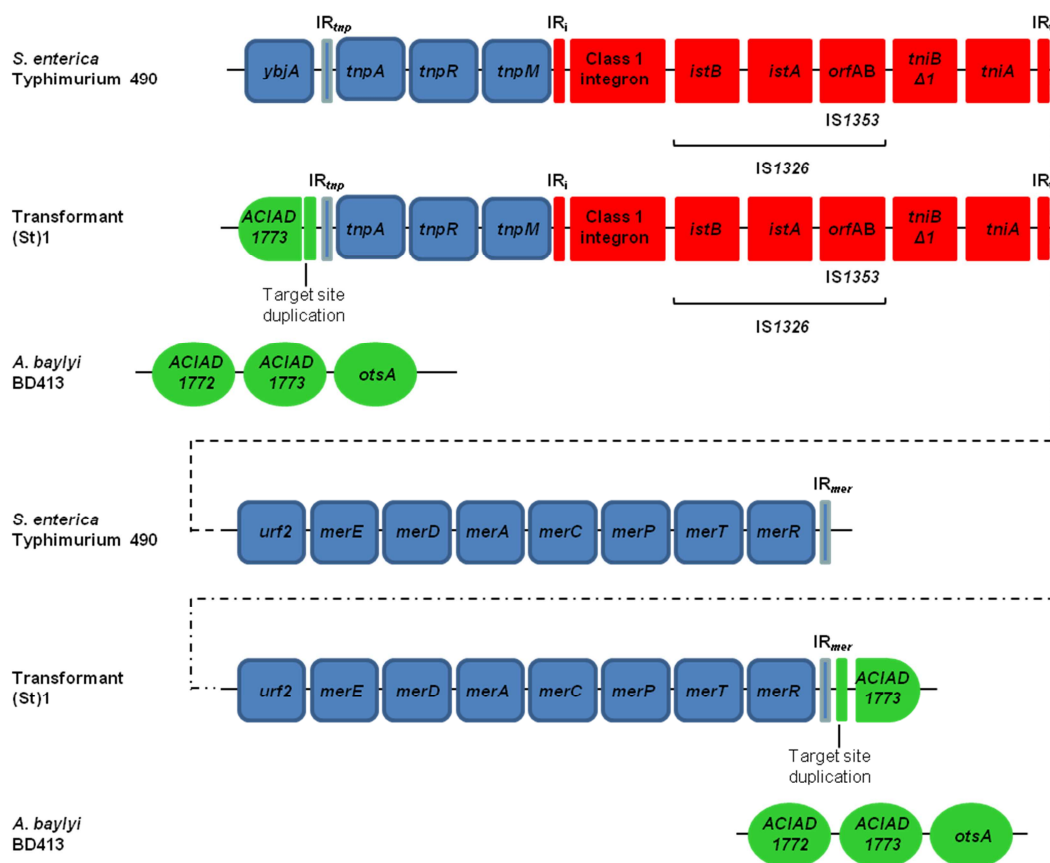


Figure 14. DNA fragment acquired by transformant *A. baylyi* (St)1 from *S. enterica* serovar Typhimurium 490. The class 1 integron includes the *intI1*, two gene cassettes, and the *qacΔE*, *sull* and *orf5* genes.

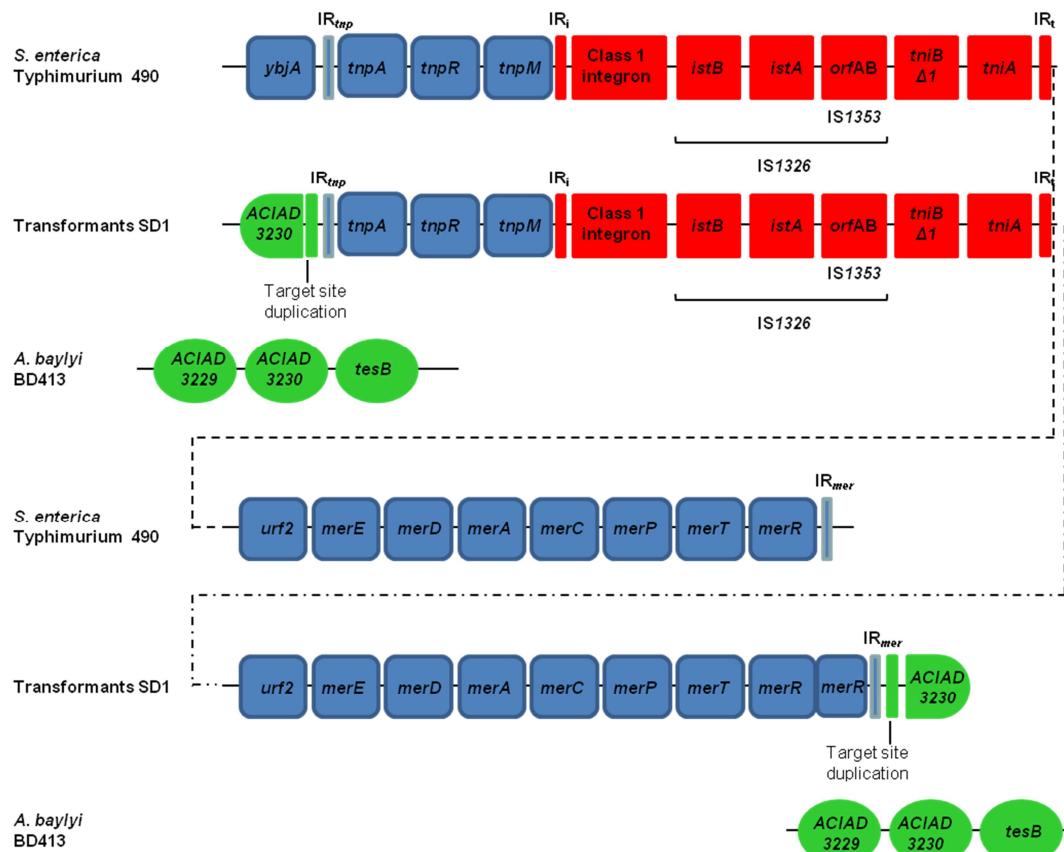


Figure 15. DNA fragment acquired by transformant *A. baylyi* SD1 from *S. enterica* serovar Typhimurium 490. The class 1 integron includes the *intl1*, two gene cassettes, and the *qac* ΔE , *sull* and *orf5* genes.

Whole-genome sequence analysis of *A. baylyi* transformant (St)3 revealed that the integron had been transferred as part of the Tn21-like transposon. In total, this transformant acquired around 23 000 bp. The transposon and a few neighbouring genes underwent rearrangements, when compared with its donor, *S. enterica* serovar Typhimurium 490. In (St)3, the *tnpR*, *tnpA*, *ybjA*, *catA1* and IS1 genes were located at the 3'-CS region, next to the *mer* operon, whereas in the previously described Tn21 sequences, they were located in the 5'-CS region (Figure 16). The rearrangement was confirmed by sequencing as well as by PCR. The Tn21-like transposon might have been acquired by the transformant by transposition, followed by a rearrangement in its genes, deleting the transposition signature. The incorporated DNA was found in the transformant chromosome, in a region containing a 54 000 bp stretch of DNA not present in

the published *A. baylyi* ADP1 strain. This DNA stretch has not been described before, and appears to be a prophage region, inserted in the ACIADtRNASer₃₄ gene.

DNA sequencing by primer walking also showed that transformant (St)2 acquired the integron embedded in the Tn21-like transposon, without rearrangements in this case.

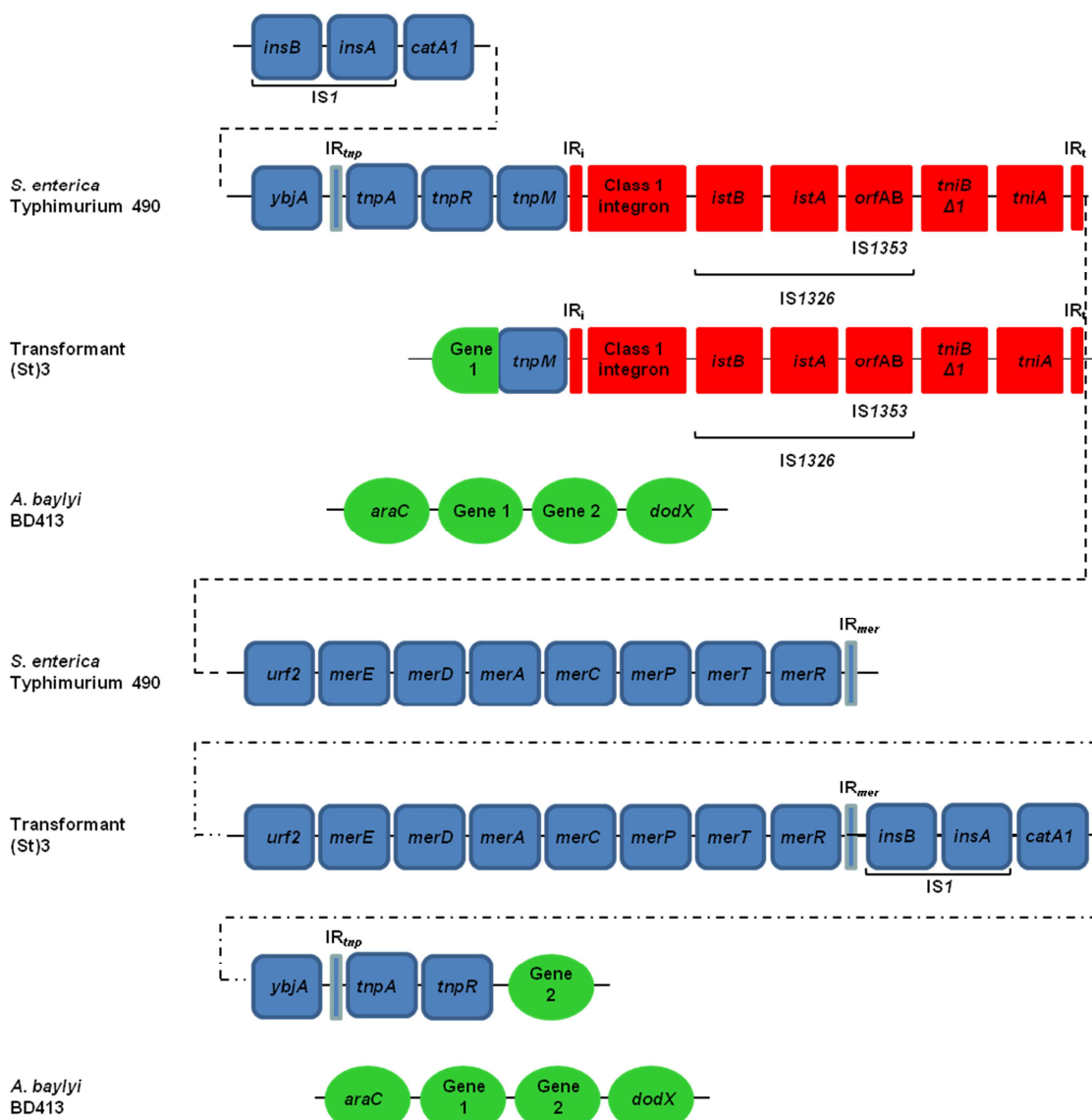


Figure 16. DNA fragment acquired by transformant *A. baylyi* (St)3 from *S. enterica* serovar Typhimurium 490. The class 1 integron includes the *int11*, two gene cassettes, and the *qacΔE*, *sull* and *orf5* genes. Gene 1 – gene coding for a bacterial regulatory, tetR family protein; Gene 2 – gene coding for a flavodoxin-like fold family protein.

2. Interspecies transfer of class 1 integrons into a transposition-module containing *A. baylyi*

MITEs are expected to be able to transpose when a transposase is provided *in trans* (Delihias, 2008); *A. baylyi* possesses some transposases in its genome (Barbe et al., 2004). Transfer of the class 1 integron of *A. baumannii* 65FFC, which is flanked by two MITE-like structures, to *A. baylyi* BD413 was not shown to occur in our experiments. We concluded that the transposases present in the *A. baylyi* are not expressed or do not recognize the IR of this MITEs and, as a consequence, do not promote the mobilization of the structure formed by the MITE-integron-MITE of *A. baumannii* 65FFC. In order to test the action of another transposase in the movement of the MITEs, the isolate *A. baylyi* SD12, containing a *tnp* module (*tnpA*, *tnpR* and *tnpM*) amplified from the strain *S. enterica* serovar Typhimurium 490, was constructed and used as recipient in transformation assays with DNA from *A. baumannii* 65FFC as donor.

No transformants were obtained in these transformation assays, where CTX 50 µg/ml was used in the transformant selective plates.

3. Interspecies transfer of resistance traits into wild-type *A. baylyi*

Natural transformation of *A. baylyi* BD413 was done with three different multidrug resistant wild-type bacteria.

3.1 Transformation frequency

Natural transformation of *A. baylyi* BD413 by the multidrug resistant isolates *E. coli* K71-77, *K. pneumoniae* K66-45 and *P. aeruginosa* K34-73 occurred at frequencies ranging from 4.4×10^{-9} to 2.1×10^{-7} (Table 10) transformants per recipient over a 24 hrs period.

Table 10. Natural transformation of *A. baylyi* BD413 with total DNA from different multidrug resistant isolates.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients
<i>A. baylyi</i> BD413	<i>E. coli</i> K71-77	$(1.0 \pm 0.6) \times 10^2$	$(4.7 \pm 0.9) \times 10^8$	2.1×10^{-7}
	<i>K. pneumoniae</i> K66-45	$(2.2 \pm 3.4) \times 10^0$	$(4.9 \pm 1.0) \times 10^8$	4.4×10^{-9}
	<i>P. aeruginosa</i> K34-73	$(3.3 \pm 4.7) \times 10^1$	$(4.8 \pm 0.7) \times 10^8$	7.0×10^{-8}

3.2 Phenotypic characterization of *A. baylyi* transformants

Transformants obtained with DNA from *P. aeruginosa* K34-73 did not show relevant changes in the antimicrobial susceptibility profile. Transformants obtained with the other two donors, *E. coli* K71-77 and *K. pneumoniae* K66-45, showed altered antimicrobial susceptibility to different antibiotics, including the one used for selection (gentamicin), but also antibiotics belonging to the same group (for example, tobramycin) or to unrelated groups (for example, sulphamethoxazole) (Table 11). This shows that transformation can contribute to the transfer of a variety of resistance traits in only one event, which is independent of the genetic relatedness of the species involved.

The transformants were not further characterized.

Table 11. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CN	CTX	SX	TOB
<i>A. baylyi</i> BD413	1.0	0.064	1.5	16	0.5
<i>E. coli</i> K71-77	> 256	> 1024	> 256	> 1024	> 256
<i>A. baylyi</i> (EclI)1	3	>256	6	256	16
(EclI)2	3	64	6	256	64
(EclI)3	3	> 256	6	256	128
(EclI)4	3	> 256	6	256	12
<i>K. pneumoniae</i> K66-45	> 256	> 1024	> 256	> 1024	> 256
<i>A. baylyi</i> (Kp)1	3	> 256	6	> 1024	> 256
(Kp)2	3	> 256	6	> 1024	> 256

^a For each transformant, the name is given by the code (donor)isolate number. The *A. baylyi* BD413 strain was the recipient. EclI - *Escherichia coli* K71-77; Kp – *Klebsiella pneumoniae* K66-45.

Antibiotics: AM – ampicillin; CN – gentamicin; CTX – cefotaxime; SX – sulphamethoxazole; TOB – tobramycin.

4. Interspecies transfer of integrons into the *A. baylyi* *recBCD sbcCD* double mutant

Natural transformation of *A. baylyi* SD9 was done with one class 1 integron-carrying wild-type donor bacterium.

4.1 Transformation frequency

As some of the observed interspecies integron transfers have taken place through transposition, a transient existence of a linear dsDNA intermediate in the cytoplasm is assumed. Such intermediates are thought to be substrates for the dsDNA-attacking exonuclease RecBCD (Kickstein et al., 2007; Dillingham and Kowalczykowski, 2008), and recent observations suggest that RecBCD also removes dsDNA-intermediates occurring during transformation (K. Harms et al., unpublished data). Thus, we constructed the *A. baylyi* strain SD9, in which we deleted the *recBCD* operon [and the RecBCD suppressor genes *sbcCD* to obtain a wild-type-like viability; (Harms and Wackernagel, 2008)] and

employed it as recipient in transformation experiments with *S. enterica* serovar Typhimurium 490 as donor. The use of this strain did not reflect an increase in the transformation frequency when compared with the wild-type *A. baylyi* BD413 (Table 12).

Table 12. Natural transformation of *A. baylyi* SD9 with class 1 integron-containing total DNA from *S. enterica* serovar Typhimurium 490.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients
<i>A. baylyi</i> SD9	<i>S. enterica</i> Typhimurium 490	$(2.0 \pm 1.5) \times 10^0$	$(1.7 \pm 0.1) \times 10^8$	1.1×10^{-8}

4.2 Genotypic characterization of *A. baylyi* transformants

Among the 22 tested transformants by PCR for detection of class 1 integrons, only three showed acquisition of the class 1 integron from the *S. enterica* serovar Typhimurium 490. The low transformation frequencies and low number of transformants that acquired a class 1 integron obtained with the strain SD9 do not suggest that RecBCD (and/or SbcCD) protects transformable cells from transposon activity initiated by linear double-stranded DNA present in the cytoplasm.

5. Interspecies transfer of integrons into an *A. baylyi* competence mutant

The competence mutant strain *A. baylyi* KOC4 was constructed by deletion of the competence operon *comFECB*, which is essential for natural transformation in *A. baylyi* (Bacher et al., 2006). Natural transformation assays with the *A. baylyi* KOC4 (Δ *comFECB*) as a recipient and DNA from *A. baumannii* 064 as donor DNA did not yield any transformants, as expected. This result reinforces

that the transformants obtained in the other assays were obtained by natural transformation.

6. Interspecies transfer of integrons/gene cassettes into class 1 integron-containing *A. baylyi*

Natural transformation of three different class 1 integron-containing *A. baylyi* strains, SD2, RAM and KOI, was performed with eight, four and five class 1 integron-carrying wild-type bacteria, respectively.

6.1 Recipient *A. baylyi* SD2

6.1.1 Transformation frequency

To determine the impact of integrons resident in the recipient bacteria on the overall transformation efficiencies, we exposed class 1 integron-containing bacteria to DNA carrying class 1 integrons of different compositions. Interspecies transformation of class 1 integron-containing *A. baylyi* SD2 recipient cells was 10- to 100-fold more efficient than interspecies transformation of wild-type *A. baylyi* BD413 cells (Table 13).

Table 13. Natural transformation of *A. baylyi* SD2 with class 1 integron-containing total DNA from different sources.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients
<i>A. baylyi</i> SD2	<i>A. baumannii</i> 65FFC	$(1.6 \pm 0.3) \times 10^5$	$(3.0 \pm 0.2) \times 10^8$	5.3×10^{-4}
	<i>C. freundii</i> C16R385	$(6.7 \pm 8.7) \times 10^4$	$(9.1 \pm 1.1) \times 10^8$	7.4×10^{-5}
	<i>E. cloacae</i> C2R371	$(2.9 \pm 0.2) \times 10^4$	$> (1.0 \pm 0.6) \times 10^9$	$< 2.9 \times 10^{-5}$
	<i>E. coli</i> C10R379	$(1.2 \pm 0.7) \times 10^4$	$(9.9 \pm 1.4) \times 10^8$	1.2×10^{-5}
	<i>E. fergusonii</i> AS041A2	$(3.1 \pm 0.2) \times 10^4$	$> (1.3 \pm 0.6) \times 10^9$	$< 2.4 \times 10^{-5}$
	<i>P. aeruginosa</i> SM	$(4.9 \pm 1.0) \times 10^3$	$(5.2 \pm 0.9) \times 10^8$	9.4×10^{-6}
	<i>S. enterica</i> Rissen 486	$(9.4 \pm 3.9) \times 10^3$	$(4.7 \pm 0.2) \times 10^8$	2.0×10^{-5}
	<i>S. enterica</i> Typhimurium 490	$(2.0 \pm 0.4) \times 10^4$	$(5.3 \pm 1.2) \times 10^8$	3.7×10^{-5}

6.1.2 Phenotypic characterization of *A. baylyi* transformants

Selected transformants were submitted to the antimicrobial susceptibility testing. All the tested transformants obtained from transformation of the recipient *A. baylyi* SD2, which contained a class 1 integron with the gene cassette *aadB*, associated with gentamicin, kanamycin and tobramycin resistance, by total DNA containing different class 1 integrons, lost the corresponding aminoglycoside resistance profile. In addition, the transformants acquired resistance to the antibiotics encoded by the gene cassettes of the correspondent donor bacteria (Table 14).

Table 14. Minimal Inhibitory Concentration (MIC; $\mu\text{g/ml}$) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baylyi</i> SD2	0.75	3	16	4	96	3	> 1024
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	n.d.	> 1024
<i>A. baylyi</i> SD3	8	> 256	n.d.	> 256	1.0	n.d.	> 1024
[SD2](Ab1)1	8	> 256	n.d.	> 256	1.0	n.d.	> 1024
[SD2](Ab1)2	8	> 256	n.d.	> 256	1.0	n.d.	> 1024

(continue)

Table 14 (continued)

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>C. freundii</i> C16R385	n.d.	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> [SD2](Cf)1	n.d.	n.d.	n.d.	n.d.	1.5	64	> 1024
[SD2](Cf)2	n.d.	n.d.	n.d.	n.d.	1.5	96	> 1024
[SD2](Cf)3	n.d.	n.d.	n.d.	n.d.	1.5	16	> 1024
<i>E. cloacae</i> C2R371	n.d.	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> [SD2](Eclo)1	n.d.	n.d.	n.d.	n.d.	1.5	> 256	> 1024
[SD2](Eclo)2	n.d.	n.d.	n.d.	n.d.	1.0	> 256	> 1024
[SD2](Eclo)3	n.d.	n.d.	n.d.	n.d.	1.0	96	> 1024
<i>E. coli</i> C10R379	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	> 1024
<i>A. baylyi</i> [SD2](Ecl)1	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	> 1024
[SD2](Ecl)2	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	> 1024
[SD2](Ecl)3	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	> 1024
<i>E. fergusonii</i> AS041A2	n.d.	n.d.	n.d.	n.d.	n.d.	8	> 1024
<i>A. baylyi</i> [SD2](Ef)1	n.d.	n.d.	n.d.	n.d.	1.5	> 1024	> 1024
[SD2](Ef)2	n.d.	n.d.	n.d.	n.d.	1.5	> 1024	> 1024
[SD2](Ef)3	n.d.	n.d.	n.d.	n.d.	1.5	96	> 1024
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> SD6	> 256	n.d.	1.0	n.d.	12	256	> 1024
[SD2](Ps)1	16	n.d.	0.5	n.d.	2	24	> 1024
[SD2](Ps)2	> 256	n.d.	1.5	n.d.	24	512	> 1024
<i>S. enterica</i> Rissen 486	> 256	n.d.	n.d.	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> SD5	1.5	n.d.	n.d.	n.d.	1.0	24	> 1024
[SD2](Sr)1	1.0	n.d.	n.d.	n.d.	1.0	96	> 1024
[SD2](Sr)2	1.0	n.d.	n.d.	n.d.	1.0	64	> 1024
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> SD4	8	n.d.	n.d.	n.d.	1.0	16	> 1024
[SD2](St)1	16	n.d.	n.d.	n.d.	1.0	96	> 1024
[SD2](St)2	4	n.d.	n.d.	n.d.	1.0	16	> 1024

^a For each *A. baylyi* transformant, the name is given by the code [recipient](donor)isolate number. Abl – *Acinetobacter baumannii* 65FFC; Cf – *Citrobacter freundii* C16R385; Eclo – *Enterobacter cloacae* C2R371; Ecl – *Escherichia coli* C10R379; Ef – *Escherichia fergusonii* AS041A2; Ps – *Pseudomonas aeruginosa* SM; Sr – *Salmonella enterica* serovar Rissen 486; St – *Salmonella enteric* serovar Typhimurium 490.

Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole.

n.d. – not determined.

6.1.3 Genotypic characterization of *A. baylyi* transformants

Transformants with changed antimicrobial susceptibility profiles were subject of PCR targeting the class 1 integron. The size of the obtained PCR-product (Figure 17) indicated that the class 1 integron-containing transformant carried the integron of the donor, instead of the integron of the recipient.

The changes in antimicrobial susceptibility profiles (Table 14) and the obtained fragment sizes by integron-specific PCR assays (Figure 17) suggested replacement of the gene cassettes rather than the accumulation of additional integrons or gene cassettes in all tested transformants (76 out of 76); homologous recombination seems to be the involved mechanism (Figure 18). Loss of the *aadB* gene in the transformants, due to the substitution of the gene cassettes from the donor, was confirmed by PCR, where no amplification of the gene was obtained.

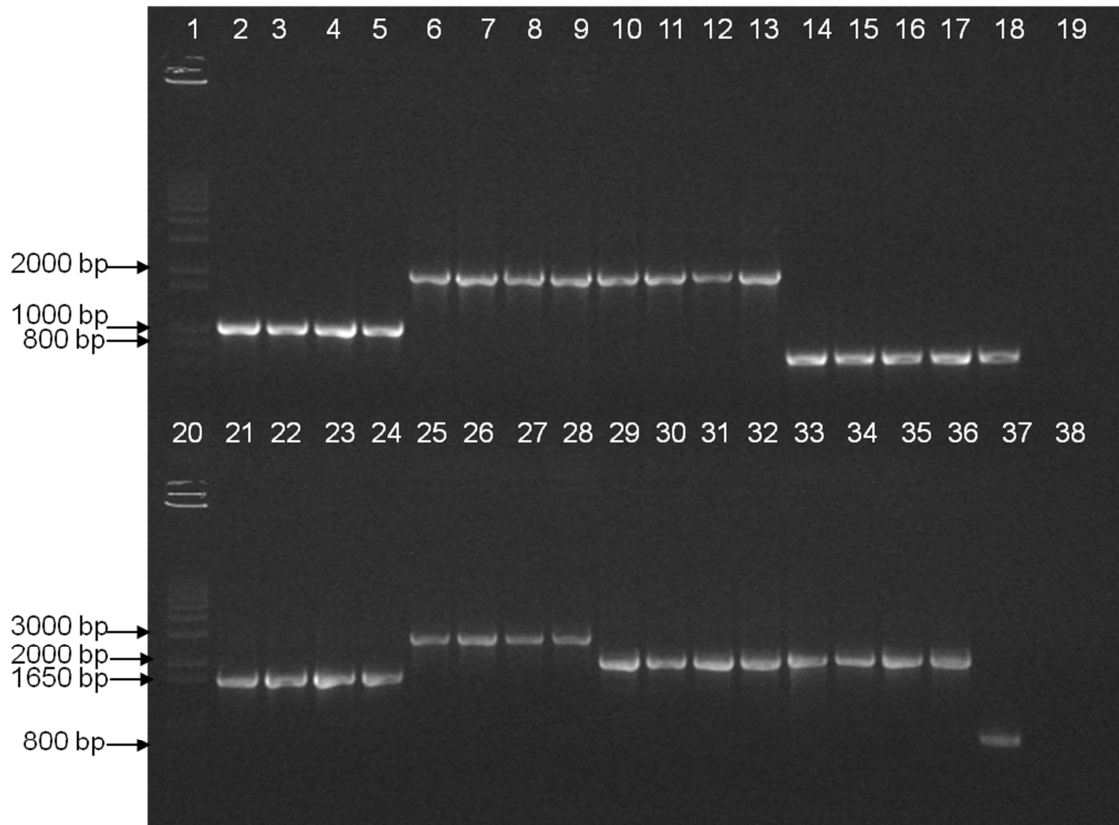


Figure 17. Agarose gel electrophoresis of class 1 integron PCR-products. Lane 1 – 1 Kb Plus DNA ladder (Invitrogen); 2 – *A. baumannii* 65FFC (donor strain); 3-5 – transformants from *A. baumannii* 65FFC, SD3, [SD2](Abl)1 and [SD2](Abl)2, respectively; 6 – *C. freundii* C16R385; 7-9 – transformants from *C. freundii* C16R385, [SD2](Cf)1, [SD2](Cf)2, [SD2](Cf)3, respectively; 10 – *E. cloacae* C2R371; 11-13 – transformants from *E. cloacae* C2R371, [SD2](Eclo)1, [SD2](Eclo)2, [SD2](Eclo)3, respectively; 14 – *E. coli* C10R379; 15-17 – transformants from *E. coli* C10R379, [SD2](Ec)1, [SD2](Ec)2, [SD2](Ec)3, respectively; 18 – transformant SD2 (recipient bacterium); 19 – *A. baylyi* BD413 (negative control); 20 – 1 Kb Plus DNA ladder (Invitrogen); 21 – *E. fergusonii* AS041A2; 22-24 – transformants from *E. fergusonii* AS041A2, [SD2](Ef)1, [SD2](Ef)2, [SD2](Ef)3, respectively; 25 – *P. aeruginosa* SM (donor strain); 26-28 – transformants from *P. aeruginosa* SM, SD6, [SD2](Ps)1, [SD2](Ps)2, respectively; 29 – *S. enterica* serovar Rissen 486 (donor strain); 30-32 – transformants from *S. enterica* serovar Rissen 486, SD5, [SD2](Sr)1, [SD2](Sr)2, respectively; 33 – *S. enterica* serovar Typhimurium 490 (donor strain); 34-36 – transformants from *S. enterica* serovar Typhimurium 490, SD4, [SD2](St)1, [SD2](St)2, respectively; 37 – transformant SD2 (recipient bacterium); 38 – *A. baylyi* BD413 (negative control).

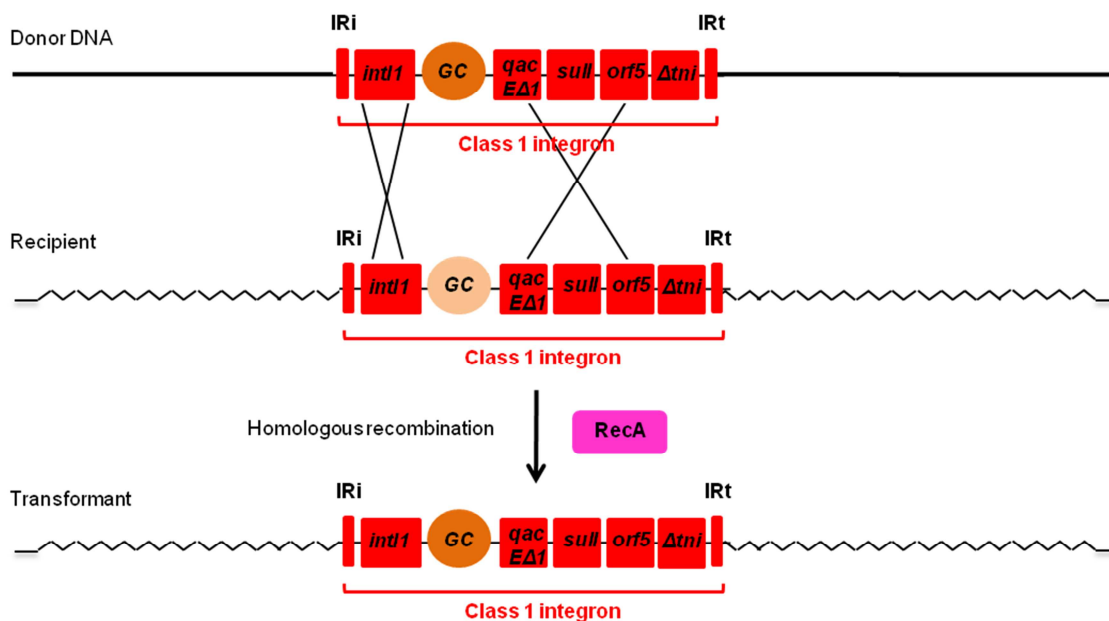


Figure 18. Schematic representation of the horizontal acquisition by substitution of gene cassettes by homologous recombination between the conserved regions of class 1 integrons.

In the case of the transformants obtained with *E. coli* C10R379 as donor DNA, the distinction of the class 1 integron was not possible based on the size of the PCR-product as both the donor and the recipient carry an integron with a variable region with similar size. However, replacement of the gene cassette of the recipient by the one from the donor can be inferred from the antimicrobial susceptibility profile and from the loss of the *aadB* gene as confirmed by PCR. For class 1 integrons containing more than one gene cassette, PCRs with a primer that binds in the class 1 integrase and primers that bind in the gene cassette present in the last position of the cassette array in the donor were done to determine if rearrangements of the gene cassettes occurred during or after the transformation. The size of the amplification product was the same for the donor and the transformants, which means that rearrangements of gene cassettes within the integrons did not occur among the analysed transformants.

6.1.4 Expression of the class 1 integrase gene in *A. baylyi* SD2

As rearrangement of gene cassettes was not detected in the obtained transformants in transformation assays with *A. baylyi* SD2 as recipient, the expression of the *intl1* gene was determined by RT-PCR. Expression of this gene was not detected (Figure 19). Lack of amplification of the *intl1* and 16S rDNA genes in the RNA sample confirmed the absence of DNA contamination, and amplification of the 16S rDNA gene confirmed the successful cDNA synthesis.

Absence or low-expression of the integrase gene is assumed from the RT-PCR results, which might explain the absence of observable recombination of gene cassettes among the number of transformants examined.

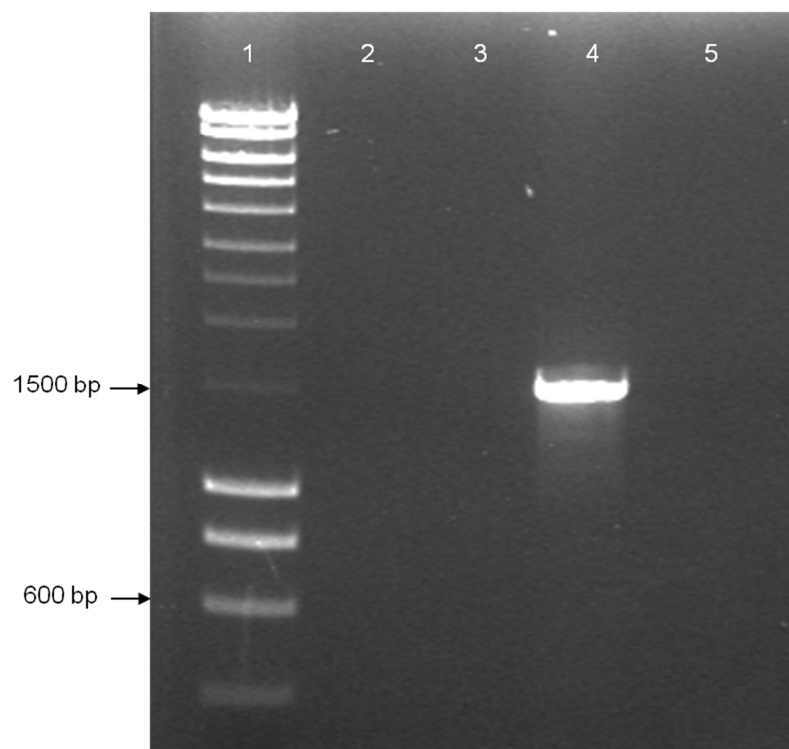


Figure 19. RT-PCR of the class 1 integrase gene in transformant *A. baylyi* SD2. Lane 1 – SmartLadder (Eurogentec); 2-3 – PCR targeting the *intl1* gene, 2 – cDNA; 3 – RNA; 4-5 – PCR targeting the 16S rDNA gene, 4 – cDNA; 5 – RNA.

6.2 Recipient *A. baylyi* RAM

6.2.1 Transformation frequency

The replacement of gene cassettes in transformation of *A. baylyi* SD2 with integron-containing DNA from different bacterial sources rather than accumulation of cassettes lead us to suggest an involvement of homologous recombination in this exchange of the cassette arrays. To test this hypothesis, an *A. baylyi* *recA*-deficient recipient strain, RAM, was constructed and used as recipient in transformation assays. Transformation frequencies of the *A. baylyi* RAM ($\Delta recA$) were in the order of 10^{-9} - 10^{-10} (Table 15), which means it was between 10^4 - to 10^5 -fold lower when compared to the *A. baylyi* *recA*-proficient recipient strain SD2. This result strongly supports homologous recombination as the mechanism responsible for efficient substitutive recombination of the gene cassettes.

Table 15. Natural transformation of *A. baylyi* RAM with class 1 integron-containing total DNA from different sources.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU)	Transformants per recipients
<i>A. baylyi</i> RAM	<i>A. baumannii</i> 65FFC	$(5.4 \pm 2.0) \times 10^0$	$> 1.7 \times 10^9$	$< 3.3 \times 10^{-9}$
	<i>P. aeruginosa</i> SM	$(7.5 \pm 7.6) \times 10^1$	$> 1.7 \times 10^9$	$< 4.6 \times 10^{-10}$
	<i>S. enterica</i> Rissen 486	$(2.1 \pm 3.3) \times 10^0$	$> 1.7 \times 10^9$	$< 1.3 \times 10^{-9}$
	<i>S. enterica</i> Typhimurium 490	$(1.9 \pm 0.8) \times 10^0$	$> 1.7 \times 10^9$	$< 1.1 \times 10^{-9}$

6.2.2 Phenotypic characterization of *A. baylyi* transformants

Although the number of acquired transformants was lower than in previous assays with the *recA*-proficient recipient strain SD2, a few obtained from transformation of RAM were further characterized. Altered antimicrobial susceptibility consistent with the donor class 1 integron acquisition by the

transformants was also seen (Table 16). Exception was seen for transformants obtained from DNA of *S. enterica* serovar Rissen 486, in which the MICs of the transformants were similar with the MICs of the recipient.

Table 16. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baylyi</i> RAM	1.5	1.5	16	1.5	24	3	> 1024
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	n.d.	> 1024
<i>A. baylyi</i> [RAM](Abl)1	4	> 256	n.d.	64	1.0	n.d.	> 1024
[RAM](Abl)2	4	> 256	n.d.	64	1.0	n.d.	> 1024
[RAM](Abl)3	4	> 256	n.d.	64	1.0	n.d.	> 1024
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> [RAM](Ps)1	> 256	n.d.	0.75	n.d.	6	384	> 1024
[RAM](Ps)2	48	n.d.	0.25	n.d.	2	32	> 1024
[RAM](Ps)3	32	n.d.	0.25	n.d.	2	32	> 1024
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> [RAM](St)1	12	n.d.	n.d.	n.d.	0.5	24	> 1024
[RAM](St)2	12	n.d.	n.d.	n.d.	0.5	16	> 1024
[RAM](St)3	12	n.d.	n.d.	n.d.	0.5	24	> 1024

^a For each *A. baylyi* transformant, the name is given by the code [recipient](donor)isolate number. Abl – *Acinetobacter baumannii* 65FFC; Ps – *Pseudomonas aeruginosa* SM; St – *Salmonella enterica* serovar Typhimurium 490.

Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole.
n.d. – not determined.

6.2.3 Genotypic characterization of *A. baylyi* transformants

Specific class 1 integron PCR in selected transformants showed acquisition of the donor integron in transformants where the susceptibility profile suggested so (22 out of 33), whereas maintenance of the recipient integron was seen in transformants that did not show changes in the MIC pattern (11 out of 33).

6.3 Recipient *A. baylyi* KOI

6.3.1 Transformation frequency

To investigate whether the integrase was required for gene cassettes acquisition or affected transformation efficiencies, the experiments, done with the recipient *A. baylyi* SD2, were repeated using the $\Delta int1$ integron-carrying *A. baylyi* strain KOI as a recipient. The resulting transformation frequencies (Table 16) were similar to those obtained with the recipient (SD2) harbouring the complete class 1 integrase gene. This also supports the involvement of homologous recombination in the cassette arrays integration.

The lack of an effect of the *int1*-encoded integrase in the recombination process was also seen when the transformant *A. baylyi* KOI(Ps)1, containing an inactive integrase, was used as donor DNA (Table 17); thus excluding a role of the integrase in the acquisition of gene cassettes in *A. baylyi* recipients already containing a class 1 integron.

Table 17. Natural transformation of *A. baylyi* KOI with class 1 integron-containing total DNA from different sources.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients
<i>A. baylyi</i> KOI	<i>A. baumannii</i> 65FFC	$(3.2 \pm 4.8) \times 10^5$	$> (1.3 \pm 0.6) \times 10^9$	$< 2.5 \times 10^{-4}$
	<i>P. aeruginosa</i> SM	$(3.5 \pm 0.9) \times 10^4$	$> 1.7 \times 10^9$	$< 2.1 \times 10^{-5}$
	<i>S. enterica</i> Rissen 486	$(2.7 \pm 0.2) \times 10^2$	$> 1.7 \times 10^9$	$< 1.7 \times 10^{-7}$
	<i>S. enterica</i> Typhimurium 490	$(7.3 \pm 4.4) \times 10^4$	$> 1.7 \times 10^9$	$< 4.4 \times 10^{-5}$
	<i>A. baylyi</i> KOI(Ps)1	$(2.9 \pm 0.3) \times 10^6$	$> 1.7 \times 10^9$	$< 1.8 \times 10^{-3}$

6.3.2 Phenotypic characterization of *A. baylyi* transformants

Changes in the antimicrobial susceptibility profile of the transformants were consistent with the acquisition of the class 1 integron from the donor bacterium (Table 18).

Table 18. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baylyi</i> KOI	2	1.5	24	1.5	32	3	> 1024
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	n.d.	> 1024
<i>A. baylyi</i> [KOI](Abl)1	6	> 256	n.d.	> 256	1.5	n.d.	> 1024
[KOI](Abl)2	6	> 256	n.d.	> 256	1.5	n.d.	> 1024
[KOI](Abl)3	6	> 256	n.d.	> 256	1.5	n.d.	> 1024
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> [KOI](Ps)1	48	n.d.	0.75	n.d.	3	128	> 1024
[KOI](Ps)2	> 256	n.d.	0.75	n.d.	4	192	> 1024
[KOI](Ps)3	> 256	n.d.	3	n.d.	48	> 1024	> 1024
<i>S. enterica</i> Rissen 486	> 256	n.d.	n.d.	n.d.	n.d.	> 1024	> 1024
<i>A. baylyi</i> [KOI](Sr)1	3	n.d.	n.d.	n.d.	1.0	> 1024	> 1024
[KOI](Sr)2	3	n.d.	n.d.	n.d.	1.0	> 1024	> 1024
[KOI](Sr)3	2	n.d.	n.d.	n.d.	1.0	> 1024	> 1024
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	192	> 1024
<i>A. baylyi</i> [KOI](St)1	16	n.d.	n.d.	n.d.	1.0	48	> 1024
[KOI](St)2	> 256	n.d.	n.d.	n.d.	1.0	> 1024	> 1024
[KOI](St)3	> 256	n.d.	n.d.	n.d.	1.0	> 1024	> 1024

^a For each *A. baylyi* transformant, the name is given by the code [recipient](donor)isolate number. Abl – *Acinetobacter baumannii* 65FFC; Ps – *Pseudomonas aeruginosa* SM; Sr – *Salmonella enterica* serovar Rissen 486; St – *Salmonella enterica* serovar Typhimurium 490. Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole. n.d. – not determined.

6.3.3 Genotypic characterization of *A. baylyi* transformants

PCR targeting the amplification of the variable region of class 1 integrons in 73 transformants showed that 70 acquired the entire gene cassette composition from the integron of the donor bacteria (Figure 20). Further nucleotide analysis of the *intl1* region of these transformants revealed that the inactivated *intl1* region had been replaced with a functional copy from the donor bacteria in four out of 10 transformants after exposure to DNA from *A. baumannii* 65FFC, in 17 out of 21 from *P. aeruginosa* SM, in 10 out of 21 from *S. enterica* Rissen 486 and in 14 out of 21 from *S. enterica* Typhimurium 490. The remaining integrons

maintained the inactive integrase of the recipient, though the cassette array has been replaced. These results demonstrate that gene cassettes are not acquired by site-specific recombination at the *attI1* site catalysed by the class 1 integrase.

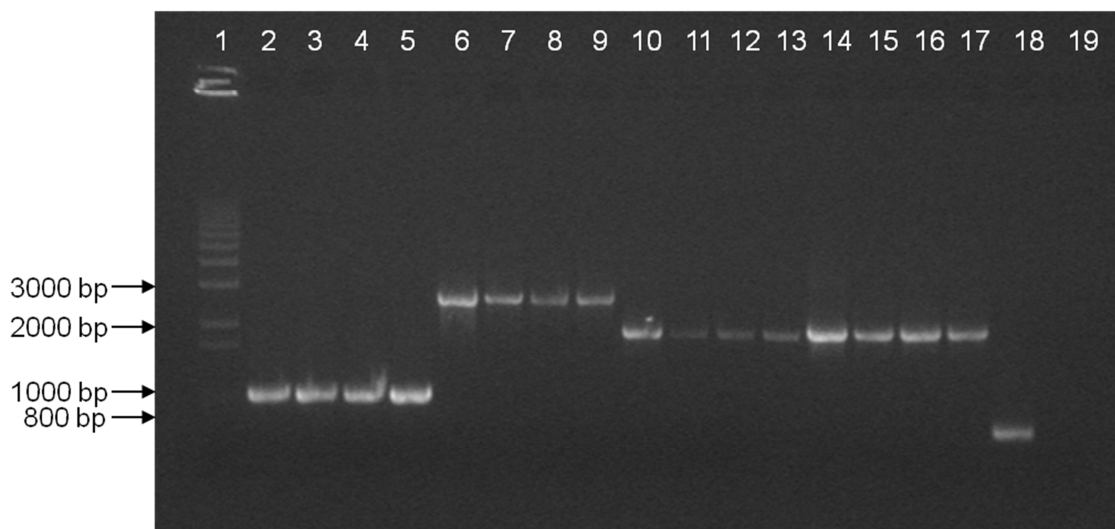


Figure 20. Agarose gel electrophoresis of class 1 integron PCR-products. Lane 1 – 1 Kb Plus DNA ladder (Invitrogen); 2 – *A. baumannii* 65FFC (donor strain); 3-5 – transformants from *A. baumannii* 65FFC, [KOI](Abl)1, [KOI](Abl)2, [KOI](Abl)3, respectively; 6 – *P. aeruginosa* SM (donor strain); 7-9 – transformants from *P. aeruginosa* SM, [KOI](Ps)1, [KOI](Ps)2, [KOI](Ps)3, respectively; 10 – *S. enterica* serovar Rissen 486 (donor strain); 11-13 – transformants from *S. enterica* serovar Rissen 486, [KOI](Sr)1, [KOI](Sr)2, [KOI](Sr)3, respectively; 14 – *S. enterica* serovar Typhimurium 490 (donor strain); 15-17 – transformants from *S. enterica* serovar Typhimurium 490, [KOI](St)1, [KOI](St)2, [KOI](St)3, respectively; 18 – transformant KOI (recipient bacterium); 19 – *A. baylyi* BD413 (negative control).

7. Intraspecies transfer of integrons into wild-type *A. baylyi*

Natural transformation of *A. baylyi* BD413 was done with six class 1 integron-carrying *A. baylyi* transformant isolates.

7.1 Transformation frequency

To investigate whether acquisition of DNA in a first transformation would impact transformation efficiencies in subsequent experiments, transformation of *A. baylyi* BD413 by DNA extracted from *A. baylyi* transformants that previously acquired an integron (SD1, SD2, SD3, SD4, SD5 and SD6) was performed. Intraspecies transformation was 10- to 1000-fold more efficient than interspecies transfer of the same integrons, and similar to the transformation obtained in the positive control with the *nptII* gene as donor DNA (4.7×10^{-4}) (Table 19). The higher transformation frequency is understandable once there are an extensive DNA homology between the donor and the recipient bacteria

Table 19. Natural transformation of *A. baylyi* BD413 with class 1 integron-containing total DNA from different sources.

Recipient strain	Donor strain	Mean no. of transformants (CFU \pm SD)	Mean no. of recipients (CFU \pm SD)	Transformants per recipients
<i>A. baylyi</i> BD413	<i>A. baylyi</i> SD1	$> 3 \times 10^5$	$(6.6 \pm 0.5) \times 10^8$	$> 4.5 \times 10^{-4}$
	<i>A. baylyi</i> SD2	$(3.0 \pm 0.1) \times 10^5$	$(5.8 \pm 0.3) \times 10^8$	5.2×10^{-4}
	<i>A. baylyi</i> SD3	$(5.5 \pm 2.8) \times 10^4$	$> 1.7 \times 10^9$	$< 3.3 \times 10^{-5}$
	<i>A. baylyi</i> SD4	$(4.1 \pm 1.4) \times 10^3$	$> 1.7 \times 10^9$	$< 2.5 \times 10^{-6}$
	<i>A. baylyi</i> SD5	$(1.9 \pm 0.4) \times 10^4$	$> 1.7 \times 10^9$	$< 1.2 \times 10^{-5}$
	<i>A. baylyi</i> SD6	$(1.6 \pm 1.3) \times 10^5$	$> 1.7 \times 10^9$	$< 9.6 \times 10^{-5}$

7.2 Phenotypic characterization of *A. baylyi* transformants

All of the tested transformants showed the expected antimicrobial susceptibility profiles if acquisition of the donor class 1 integron had occurred; the various transformants showed a similar resistance profile (Table 20).

Table 20. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* transformants as determined by the Etest method.

Bacteria ^a	AM	CAZ	CN	CTX	K	SC	SX
<i>A. baylyi</i> BD413	1.0	1.5	0.064	1.5	0.38	3	16
<i>A. baylyi</i> SD2	0.75	n.d.	16	n.d.	96	n.d.	> 1024
(SD2)1	0.75	n.d.	16	n.d.	64	n.d.	> 1024
(SD2)2	0.75	n.d.	24	n.d.	64	n.d.	> 1024
(SD2)3	0.75	n.d.	16	n.d.	64	n.d.	> 1024
<i>A. baylyi</i> SD3	8	> 256	n.d.	> 256	1.0	n.d.	> 1024
(SD3)1	6	> 256	n.d.	> 256	n.d.	n.d.	> 1024
(SD3)2	6	> 256	n.d.	> 256	n.d.	n.d.	> 1024
(SD3)3	6	> 256	n.d.	> 256	n.d.	n.d.	> 1024
<i>A. baylyi</i> SD6	> 256	n.d.	1.0	n.d.	12	256	> 1024
(SD6) 1	> 256	n.d.	1.0	n.d.	n.d.	192	> 1024
(SD6) 2	> 256	n.d.	1.0	n.d.	n.d.	512	> 1024
(SD6) 3	> 256	n.d.	1.0	n.d.	n.d.	384	> 1024
<i>A. baylyi</i> SD5	1.5	n.d.	n.d.	n.d.	1.0	24	> 1024
(SD5)1	1.0	n.d.	n.d.	n.d.	n.d.	48	> 1024
(SD5)2	1.0	n.d.	n.d.	n.d.	n.d.	32	> 1024
(SD5)3	0.75	n.d.	n.d.	n.d.	n.d.	32	> 1024
<i>A. baylyi</i> SD1	6	n.d.	n.d.	n.d.	n.d.	12	> 1024
SD4	8	n.d.	n.d.	n.d.	1.0	16	> 1024
(SD1)1	6	n.d.	n.d.	n.d.	n.d.	12	> 1024
(SD1)2	4	n.d.	n.d.	n.d.	n.d.	12	> 1024
(SD1)3	6	n.d.	n.d.	n.d.	n.d.	16	> 1024
(SD4)1	6	n.d.	n.d.	n.d.	n.d.	16	> 1024
(SD4)2	4	n.d.	n.d.	n.d.	n.d.	12	> 1024
(SD4)3	6	n.d.	n.d.	n.d.	n.d.	16	> 1024

^a The transformants SD1 and SD2 were obtained in transformation of *A. baylyi* BD413 by DNA from *S. enterica* serovar Typhimurium 490 and *A. baumannii* 064, respectively. Transformants SD3, SD4, SD5 and SD6 were obtained in transformation of *A. baylyi* SD2 by DNA from *A. baumannii* 65FFC, *S. enterica* serovar Typhimurium 490, *S. enterica* serovar Rissen 486 and *P. aeruginosa* SM, respectively.
Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; SC – spectinomycin; SX – sulphamethoxazole.
n.d. – not determined.

7.3 Genotypic characterization of *A. baylyi* transformants

7.3.1 Class 1 integron targeting PCR

All the transformants with altered antimicrobial susceptibilities were tested using the class 1 integron specific PCR. All transformants were positive for integron amplification, which presented bands of the same size as in the donor bacterium (Figure 21); reflecting the transfer of the entire class 1 integron.

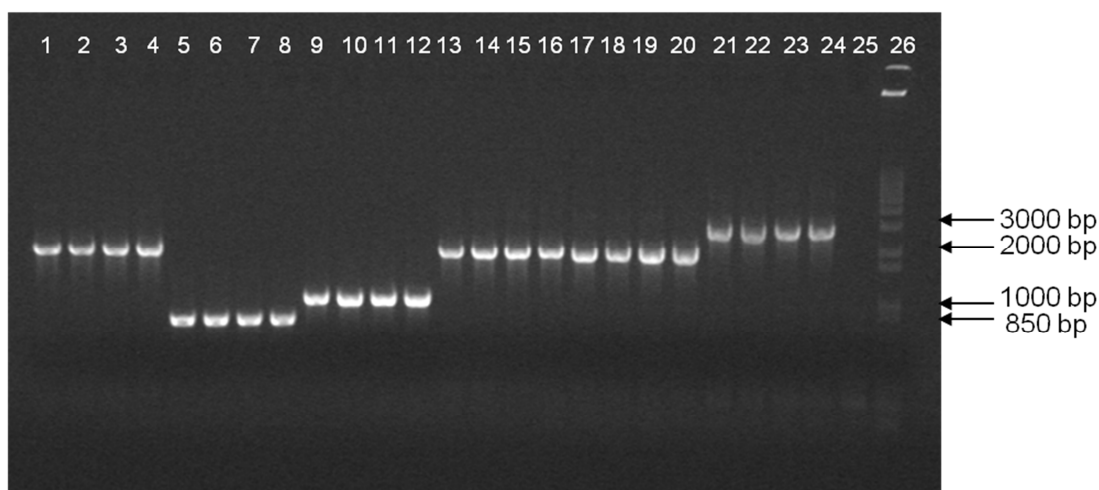


Figure 21. Agarose gel electrophoresis of class 1 integron PCR-products. Lane 1 – transformant SD1 (donor strain); 2-4 – transformants from SD1, (SD1)1, (SD1)2, (SD1)3, respectively; 5 – transformant SD2 (donor strain); 6-8 – transformants from SD2, (SD2)1, (SD2)2, (SD2)3, respectively; 9 – transformant SD3; 10-12 – transformants from SD3, (SD3)1, (SD3)2, (SD3)3, respectively; 13 – transformant SD4; 14-16 – transformants from SD4, (SD4)1, (SD4)2, (SD4)3, respectively; 17 – transformant SD5; 18-20 – transformants from SD5, (SD5)1, (SD5)2, (SD5)3, respectively; 21 – transformant SD6; 22-24 – transformants from SD6, (SD6)1, (SD6)2, (SD6)3, respectively; 25 – *A. baylyi* BD413 (recipient strain); 26 – 1 Kb Plus DNA ladder (Invitrogen).

7.3.2 DNA sequencing

Crossover junctions in the *A. baylyi* genome of selected transformants were determined by DNA sequencing. The transformant isolates (SD1)1, (SD1)2 and

(SD1)3, obtained with the donor strain *A. baylyi* SD1, acquired at least 23 500 bp of the donor DNA. The transformant isolates (SD2)1, (SD2)2 and (SD2)3, obtained with the donor *A. baylyi* SD2, obtained at least 20 000 bp of the donor DNA. The exact size of the recombining regions for these isolates remains undetermined as the donor and recipient genomes have regions with identical DNA composition in the areas flanking the DNA insertion. Extensive DNA sequencing by primer walking of six transformants suggested that the composition of the donor DNA segments acquired in the initial interspecies transformation assay were maintained after subsequent intraspecies transformation (the same as shown in Figures 9 and 14).

The conserved composition of the acquired integron and the flanking regions in six out of six tested transformants, suggested that the observed intraspecies gene transfer occurred via homologous recombination-facilitated DNA exchange between the two *A. baylyi* genomes involved.

III. Characterization of transformants with reduced susceptibility to antibiotics

Exposure of *A. baylyi* cells to the various DNA substrates used produced several transformants in which we could not identify the acquisition of a class 1 integron, or other known antimicrobial resistance genes from the donor strain. These phenotypic transformants showed a reduced susceptibility profile to some antibiotics, mainly to ampicillin and sulphonamides, when compared with the profile of the unexposed recipient bacterium (Table 21). Formation of mosaic genes is known to occur in genes related to decreased susceptibility to β -lactams and sulphonamides (Maiden, 1998), the same antibiotics that our transformants showed diminished susceptibility (Maiden, 1998). Therefore, our results suggested that exposure of competent bacteria to heterologous DNA may have led to transfer of small DNA fragments affecting the antimicrobial

resistance profile of some of the transformants, or other transferable traits not known to be involved in resistance so far.

In order to unravel the origin of such reduced antimicrobial susceptibility, the genome of one of the transformants, *A. baylyi* (Abl)L2, was fully sequenced and analysed. Additionally, and as mosaic genes are frequently found in PBP genes, part of these genes were amplified and sequenced in selected transformants with altered susceptibility to ampicillin. Mosaics involvement in the acquired reduced susceptibility of transformants to sulphonamides could not be investigated, as no genes associated with resistance to this antibiotic have been identified in *A. baylyi* (Gomez and Neyfakh, 2006).

Table 21. Minimal Inhibitory Concentration (MIC; µg/ml) of *A. baylyi* isolates obtained as determined by the Etest method.

Bacterial isolate ^a	AM	CAZ	CN	CTX	K	SX
<i>A. baylyi</i> BD413	1.0	1.5	0.064	1.5	0.38	16
<i>A. baumannii</i> 064	> 256	n.d.	> 256	n.d.	> 256	> 1024
<i>A. baylyi</i> (AbII)5	12	n.d.	0.5	n.d.	1.0	32
<i>A. baylyi</i> (AbII)6	1.0	n.d.	0.38	n.d.	> 256	48
<i>A. baylyi</i> (AbII)L1	32	n.d.	0.38	n.d.	0.5	24
<i>A. baumannii</i> 65FFC	64	> 256	n.d.	> 256	n.d.	> 1024
<i>A. baylyi</i> (Abl)L1	6	8	n.d.	8	n.d.	16
<i>A. baylyi</i> (Abl)L2	4	8	n.d.	8	n.d.	64
<i>A. baylyi</i> (Abl)L3	0.75	1.5	n.d.	1.5	n.d.	24
<i>P. aeruginosa</i> SM	> 256	n.d.	16	n.d.	n.d.	> 1024
<i>A. baylyi</i> (Ps)1	6	n.d.	0.19	n.d.	n.d.	24
<i>A. baylyi</i> (Ps)L1	6	n.d.	0.5	n.d.	n.d.	24
<i>A. baylyi</i> (Ps)L2	6	n.d.	0.38	n.d.	n.d.	24
<i>S. enterica</i> Rissen 486	> 256	n.d.	n.d.	n.d.	n.d.	> 1024
<i>A. baylyi</i> (Sr)1	4	n.d.	n.d.	n.d.	n.d.	16
<i>S. enterica</i> Typhimurium 490	> 256	n.d.	n.d.	n.d.	n.d.	> 1024
<i>A. baylyi</i> (St)L1	6	n.d.	n.d.	n.d.	n.d.	64
<i>A. baylyi</i> (St)L2	6	n.d.	n.d.	n.d.	n.d.	32

^a For each transformant, the name is given by the code (donor)isolate number. The *A. baylyi* BD413 strain was the recipient. Abl – *Acinetobacter baumannii* 65FFC; AbII – *Acinetobacter baumannii* 064; Ps – *Pseudomonas aeruginosa* SM; Sr – *Salmonella enterica* serovar Rissen 486; St – *Salmonella enterica* serovar Typhimurium 490. When the isolate number is preceded by an "L", transformants were obtained with lysate DNA as donor. Antibiotics: AM – ampicillin; CAZ – ceftazidime; CN – gentamicin; CTX – cefotaxime; K – kanamycin; and SX – sulphamethoxazole. n.d. – not determined.

1. Full genome sequence of transformant *A. baylyi* (AbI)L2

Acquisition of the class 1 integron from *A. baumannii* 65FFC by the transformant *A. baylyi* (AbI)L2 was not detected by class 1 integron specific PCR, and confirmed by the full genome sequence of this isolate. The obtained genome sequence did not reveal major DNA acquisitions as well, when compared with the published genome of *A. baylyi* ADP1.

The change in the resistance profile of isolate (AbI)L2 was therefore attempted resolved by examining other acquired nucleotide changes, in comparison with the known sequences of the genes known to be involved in intrinsic antibiotic resistance in *A. baylyi* ADP1 (GenBank accession number CR543861) – ACIAD0795, *acrB*, *ampD*, *argH*, *gph*, *gshA*, *hisF*, *mpl*, *oprM*, *pbpG* and *recD* genes (Gomez and Neyfakh, 2006); in the PBP-coding genes – ACIAD1184, *dacC*, *ftsI*, *pbpA* and *ponA* genes (the *pbpG* is already included in the previous group of genes) (GenBank accession number CR543861); and in the *ampC* gene (Beceiro et al., 2007). Again, non-synonymous nucleotide changes that could explain the reduced antimicrobial susceptibility patterns of the transformants were not identified.

2. PBP-coding genes sequencing

Internal segments of the PBP-coding genes ACIAD1184 (1258/1311 bp), *dacC* (942/1149 bp), *ftsI* (1636/1866 bp), *pbpA* (1897/2028 bp), *pbpG* (715/1059 bp) and *ponA* (2361/2559 bp) were amplified and sequenced in transformants (AbI)L1, (Sr)1, (AbII)5, (AbII)7, (AbII)8, (AbII)L2, (Ps)L1, (Ps)L2 and (Ps)L3, derived from four different donor sources and that showed significant reduced susceptibility to ampicillin (Table 21). The obtained sequences were compared with the published sequences from *A. baylyi* ADP1 (GenBank accession number CR543861), but also with the sequence we obtained from the same genes in our recipient *A. baylyi* BD413. The PBP-coding genes of the both

strains revealed to be 100 % identical (at least in the sequenced region). Neither nucleotide changes nor acquisition of DNA blocks were identified in the analysed PBP gene fragments. It must be noted that the complete length of the genes was not analysed, missing between 17 and 151 bp in the ends of the genes, and around 360 bp inside the *ponA* gene. Thus, alterations could have been acquired in the region not included in the sequencing. However, from the analyses of the full genome of *A. baylyi* (AbI)L2, formation of mosaic genes in the PBP genes with consequent altered antimicrobial susceptibility might not be the responsible mechanism for the reduced susceptibility to ampicillin. These observations suggest that the altered antimicrobial susceptibility profiles in transformants might be due to smaller recombination events that had occurred in other genes that have not been yet described as involved in resistance, or the outcome of other transferable traits of unknown mechanistic and genetic nature.

IV. Relative fitness assays

The biological cost of the acquired, integron-mediated, antibiotic resistance was determined in eight *A. baylyi* transformants, carrying altogether class 1 integrons with five different gene cassette arrays: SD3 (*bla*_{IMP-5}), (St)3, (SD1)1 and SD4 (*bla*_{OXA-30}-*aadA1*), SD5 (*dfrA12*-*aadA2*), SD6 (*aacA4*-*bla*_{PSE}-*aadA2*), and SD2 and (SD2)1 (*aadB*). It is important to have in consideration that these transformants carry in their genome at least 20 000 bp of acquired DNA.

The mean relative fitness (W) of each class 1 integron-carrying *A. baylyi* transformant was calculated as the ratio of the Malthusian parameter of each competitor. The results are interpreted based on the value of 1 for the W of the *A. baylyi* BD413; a fitness cost is conferred by the acquired DNA in transformants with $W < 1$, and a benefit is present when $W > 1$. The cost is given by the difference between the reference 1 and the W of the transformant, and can be given in percentage. Detailed results are shown in Table 22. The mean W ranged from 0.96 to 1.01 ($n = 19$ to 24 replicates) in all transformants,

except SD3; these results were not statistically significant (p between 0.138 and 0.897). Transformant SD3 showed a clear negative fitness effect, with a mean $W = 0.91$ ($n = 23$; $p = 0.001$); the cost of carriage of this class 1 integron is 9 %. The W of the transformants did not seem to be affected by the class 1 integron composition, as different gene cassette arrays conferred similar cost to the bacterium. For example, the transformants *A. baylyi* SD2, SD4 and SD5, carrying three different gene cassette arrays, have a $W = 0.99$.

Table 22. Relative fitness cost conferred by the acquisition of class 1 integron-containing DNA by different *A. baylyi* transformants.

Transformant	Replicates	W mean (\pm SD)	Fitness cost or advantage (%) ^a	Lower W	Higher W
<i>A. baylyi</i> SD3	23	0.91 (\pm 0.12)	(-) 9	0.70	1.12
<i>A. baylyi</i> (St)3	24	1.01 (\pm 0.14)	(+) 1	0.77	1.41
<i>A. baylyi</i> (SD1)1	19	1.00 (\pm 0.14)	0	0.78	1.32
<i>A. baylyi</i> SD4	20	0.99 (\pm 0.07)	(-) 1	0.82	1.08
<i>A. baylyi</i> SD5	21	0.99 (\pm 0.08)	(-) 1	0.79	1.14
<i>A. baylyi</i> SD6	20	0.96 (\pm 0.12)	(-) 4	0.72	1.19
<i>A. baylyi</i> SD2	23	0.99 (\pm 0.12)	(-) 1	0.80	1.40
<i>A. baylyi</i> (SD2)1	23	1.00 (\pm 0.13)	0	0.84	1.29

^a (-) represents a fitness cost; (+) represents a fitness advantage.

In general, no consistent major differences in fitness were observed, suggesting that the horizontal acquisitions of integrons (including the co-transferred and often extensive additional DNA regions) do not lead to immediate and severe growth inhibition of transformant cells. In addition, initial costly genes can be maintained, as initial fitness costs can be ameliorated by compensatory mutations. A recent study by Starikova and colleagues showed that the class 1 integron of *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490, free of associated transposable elements, and introduced into *A. baylyi* by natural transformation, conferred an initial fitness cost of 7 and 11 %, respectively, in the transformants. After an experimental evolution assay, the W of the transformant containing the integron from *A. baumannii* 064 was completely restored. The reduction of the fitness cost was shown to occur due to a

mutation in the class 1 integrase gene (Starikova et al., 2012). A previous stability study performed with the integron of *A. baumannii* 064 over 10 days showed that this integron and integrated gene cassette were stably maintained in the presence or absence of antibiotic selection (Munthali, 2010).

Nucleotide accession numbers

The nucleotide sequences deposited in GenBank were given the accession numbers JX235356, JF810083 and JX041889.

Discussion

HGT is a key driver of bacterial adaptation and evolution (Ochman et al., 2000; Gogarten et al., 2002; van Hoek et al., 2011). A number of studies demonstrate that a significant amount of bacterial genomes are affected by HGT events (Ochman et al., 2005; Thomas and Nielsen, 2005; Retchless and Lawrence, 2007; Kloesges et al., 2011), and antibiotic resistance-carrying MGEs represent a considerable portion of the transferred elements (Aminov, 2011). Among MGEs, class 1 integrons are considered a major vector for antibiotic resistance dissemination among bacteria, especially amongst Gram-negative species (Cambray et al., 2010). Many studies report on high prevalence of these MGEs (Martinez-Freijo et al., 1998; White et al., 2001; Segal et al., 2003; Molla et al., 2007); and the same gene cassette array, sometimes with minor nucleotide changes, is often found in different species from diverse origins.

In this study, we examined the prevalence of class 1 integrons in Gram-negative bacterial isolates from human clinical samples and animal sources, and evaluated to what extent DNA substrates and natural transformation can lead to transfer of distinct genetic elements encoding site-specific recombination mechanisms. We provide experimental data demonstrating that natural transformation can facilitate intra- and interspecies transfer of class 1 integrons and transposons, which are not limited by the genetic relatedness of the donor with the recipient host. Natural transformation has been previously suggested to be involved in the interspecies transfer of non-plasmid located class 1 integrons (Gombac et al., 2002). Acquisition of complete class 1 integrons and its flanking DNA by *A. baylyi* transformant cells was confirmed by resistance profiling, PCR-based target amplification, PFGE and Southern blot hybridization, and extensive DNA sequencing.

I. Class 1 integrons and their flanking regions

1. Prevalence and diversity of class 1 integrons

In our study, 41 % of the clinical *Acinetobacter* spp. isolates collected in two Portuguese regions between 1992 and 1999, contained a class 1 integron. High prevalence of class 1 integrons in clinical *Acinetobacter* spp. has also been reported in other studies. For instance, Gombac and colleagues (Gombac et al., 2002) reported that 44 % of the *A. baumannii* isolates, collected in six Italian hospitals between 1989 and 2000, carried a class 1 integron. Among 48 *Acinetobacter* spp. isolated from 11 countries, including Austria, Belgium, Canada, Czech Republic, France, Germany, Italy, The Netherlands, Spain, United Kingdom and United States, between 1986 and 1997, 44 %, identified at the time as *A. baumannii*, carried a class 1 integron (Koeleman et al., 2001). Class 1 integrons were also identified in 43 % of clinical *Acinetobacter* spp. isolated in a hospital in South China between 2005 and 2006; the species were *A. bereziniae* (formerly *Acinetobacter* genomic species 10) (n = 2), *Acinetobacter pittii* (formerly *Acinetobacter* genomic species 3) (n = 7), *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 13TU) (n = 18) and *A. baumannii* (n = 26) (Xu et al., 2008a; Nemeč et al., 2010; Nemeč et al., 2011). Class 1 integrons have also been reported in non-clinical *Acinetobacter* spp., isolated from freshwater of Danish trout farms, but the proportion of isolates carrying class 1 integrons (21 %) was lower than in clinical settings (Petersen et al., 2000).

All the clinical *Acinetobacter* spp. with a class 1 integron examined our study, except the *A. bereziniae* 118FFC, contained the cassette array *aacC1-orfA-orfB-aadA1*. These isolates were collected in two different regions of Portugal over a period of eight years, showing different susceptibility profiles (data not shown). Accordingly to the new nomenclature of open reading frames (*orf*) of gene cassettes proposed by Partridge *et al.* (Partridge et al., 2009), this array is

now known as *aacC1-gcuP-gcuQ-aadA1*. The naming of *orf* gene cassettes, which encode proteins with unknown function, is confusing, as the same name has been attributed to different genes and the same gene has been named differently. For instance, *orfA* has also been named *orfX* and *orf9* (Partridge et al., 2009). The same cassette array (> 98 % nucleotide identity) has been denominated *aacC1-orfA-orfB-aadA1* (GenBank accession number HM366564), *aacC1-orfP-orfQ-aadA1* (GenBank accession number CP002522), *aacC1-orf1-orf2-aadA1* (GenBank accession number AJ784787), *aacC1-orfX-orfY-aadA1a* (GenBank accession number AJ310480), *aacC1-orfX-orfQ-aadA1a* (GenBank accession number HM589045) and *aacC1-orfX-orfX'-aadA1a* (GenBank accession number AY922991). This array of gene cassettes has been found in several isolates, in different countries and in a variety of species. For example, *A. baumannii* strains carrying this class 1 integron have been isolated in hospitals in Italy (Gombac et al., 2002; Zarrilli et al., 2004), Spain (Ribera et al., 2004), United Kingdom (Turton et al., 2005), China (Xu et al., 2008a) and Australia (Mak et al., 2009), among other countries. These isolates were collected in a wide period of time, from 1989 to 2006, which also comprises the time period in which our *Acinetobacter* spp. isolates were collected. The same gene cassette array has been reported in an *A. baumannii* strain isolated from a clinical sample of a horse (Abbott et al., 2005) and later in six *E. coli* strains, also collected from hospitalized animals (Karczmarczyk et al., 2011). Besides these reports, nucleotide sequences of the same gene cassette array, from other species, have been deposited in GenBank and include *P. aeruginosa* (GenBank accession number HM366564) and *K. pneumoniae* (GenBank accession number HM589045 and GU906254). Taking this single gene cassette array as an example, it is clear that class 1 integrons are globally disseminated, and that the spread is not limited to a specific source, region, time period or bacterial species.

Although the cassette array *aacC1-orfA-orfB-aadA1* is not restricted to *Acinetobacter* spp., it seems to have a main distribution in this genus, especially in *A. baumannii* populations, as the majority of the reports are on this species. This is probably due to the insertion of this class 1 integron in larger MGEs that

are well suited for dissemination among *A. baumannii*, such as the resistance islands AbaR. In fact, this cassette array has been reported in several AbaRs, such as the AbaR7 (Post et al., 2010), AbaR3, AbaR13, AbaR16, AbaR17 and AbaR18 (Krizova et al., 2011). Extensive sequencing of the flanking regions of the class 1 integron in the isolates with the gene cassette array *aacC1-orfA-orfB-aadA1* would clarify whether all, or the majority, of them are associated with the resistance islands of *Acinetobacter*. Furthermore, sequencing of the flanking regions of the same class 1 integron found in the other genera of bacteria would also elucidate if, and possibly how, this integron was acquired by HGT from the *Acinetobacter* species.

Although *A. baumannii* is reported as the most common nosocomial pathogen of the genus *Acinetobacter*, other species also assume clinical relevance (Bergogne-Berezin and Towner, 1996; Turton et al., 2010). *A. bereziniae* is one of the species that has been associated with infections (Bergogne-Berezin and Towner, 1996; Xu et al., 2008a; Kuo et al., 2010; Lee et al., 2010; Yamamoto et al., 2012). Two strains of *A. bereziniae* isolated in China (Xu et al., 2008a), five from South Korea (Lee et al., 2010) and nine from Japan (Yamamoto et al., 2012) carried a class 1 integron. *A. bereziniae* 118FFC also carried a class 1 integron, In796, with a cassette array that has not been identified before. The gene cassette array of In796, *aacA7-bla_{VIM-2}-aacC1*, is similar with the array of In58 (GenBank accession number AF263520), with the exception that In58 contains an *aacA4* gene after the *aacC1* cassette. The nucleotide sequence of both integrons is 100 % identical in stretches of 3361 bp and 1650 bp, which include the region with the *intl1*, *aacA7*, *bla_{VIM-2}* and *aacC1* genes and the region with the *qacED1*, *sul1* and *orf5* genes, respectively. *A. bereziniae* 118FFC containing In796 is a clinical isolate collected in Portugal in 1998, and In58 was detected in the clinical strain *P. aeruginosa* RON-1 collected in France also in 1998 (Poirel et al., 2001). The similar nucleotide sequence and the array of gene cassettes suggests that In58 could be the precursor of the In796, due to the loss of the last gene cassette, which can occur in the absence of antibiotic pressure (Collis and Hall, 1992b; Rosser and Young, 1999). In796 in *A.*

bereziniae 118FFC is flanked by two MITE-like structures, and a TSD on both sides suggest acquisition of this structure by transposition. It would be interesting to determine the flanking regions of In58, and if the same MITE-like structures flank this class 1 integron and contributes to its dissemination.

In our study, class 1 integrons were detected in 14.5 % of the bacterial isolates collected from domestic (dog), food-producing (chicken and rabbit) and wild (owl) animals. Similar prevalence of class 1 integrons has been reported in gulls (15 %) (Dolejska et al., 2009) and in clinical samples collected from swine, horses, dogs and cats (13.9 %) (Kadlec and Schwarz, 2008). These animals inhabit diverse environments and their bacterial population can act as conduits for integron-borne resistance genes dissemination. Besides the fact that integrons present in these bacteria can end up in contaminating bacteria in food products, manure is often used as a fertilizer and sewage easily contact with water streams, with consequent contamination of soil, water, fruits and vegetables, and wildlife. Humans will therefore be in contact with these animal and environmental sources of integrons, and can similarly contribute to the spread of bacteria into these habitats, as the flow of bacteria and their genetic components is multidirectional. Direct exchange of resistance genes can occur between any of the bacterial strains present in human beings, domestic, companion and wild animals and environment (Stokes and Gillings, 2011). There is evidence that suggests that humans are the main source for transmission of class 1 integrons to animals, rather than the opposite (Skurnik et al., 2006; Diaz-Mejia et al., 2008). It is nevertheless often difficult to establish the directionality of the HGT events (Stokes and Gillings, 2011).

Eight of the bacterial strains isolated from food-producing animals in our study, all enterobacteria belonging to four different species (*C. braakii*, *C. freundii*, *E. cloacae* and *E. coli*) were shown to carry the same class 1 integron array, *dfrA12-orfF-aadA2*. This integron, named In27, is widespread and has been found in numerous species, from different origins and regions. This array is very common in bacteria from animal sources, and it has been identified in *K.*

pneumoniae isolated from poultry meat in Portugal (Machado et al., 2008); in *S. enterica* serovars Anatum, Saintpaul and Braenderup, collected from chicken in Ethiopia (Molla et al., 2007); in *E. coli* isolated from marten, *E. cloacae* from Eurasian badger and *Morganella morganii* isolated from kite and owl in Japan (Ahmed et al., 2007b); in *Aeromonas hydrophila* collected in USA from a pig (Poole et al., 2006); in *E. coli* from swine, horse, dog and cat origin in Germany (Kadlec and Schwarz, 2008); in *E. coli* from brackish-water fish farms in Egypt (Ishida et al., 2010); and in *S. enterica* serovar Rissen from wild boars and Bísaro pigs, collected in Portugal (Caleja et al., 2011). *Enterobacteriaceae* seems to be the major group of bacteria carrying the integron array *dfrA12-orfF-aadA2* (Stokes and Gillings, 2011), though this observation may be due to sampling bias as this group of bacteria predominate in animals. These isolates were collected from a variety of domestic, wild, zoo, food and food-producing animals, as well as food products, which may reflect the transmission of antimicrobial resistant bacteria between animals from diverse environments; especially birds can spread resistant bacteria over longer distances. Besides the animal background, the gene cassette array *dfrA12-orfF-aadA2* is also found widespread among clinical isolates, including *A. baumannii* and *P. aeruginosa* isolated in China (Gu et al., 2007); *E. coli* collected from Korea (Kang et al., 2005); strains of *Enterobacter hormaechei* subsp. *steigerwaltii* isolated in Poland (Mokracka et al., 2011); *S. enterica* collected in Spain (Garcia et al., 2011); and *E. faecalis*, *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus warneri*, undefined coagulase-negative *Staphylococcus* and *Streptococcus* spp. isolated in China (Shi et al., 2006; Xu et al., 2008b; Xu et al., 2011a; Xu et al., 2011b). An *E. coli* strain isolated from estuarine water in Portugal carried the same gene cassette array as well (Henriques et al., 2006). HGT is suggested to be involved in the spread of the class 1 integrons in most of the studies that have reported several, non-epidemiological related, bacterial isolates with the same gene cassette array (Kang et al., 2005; Shi et al., 2006; Xu et al., 2008b; Krauland et al., 2009). Further work on the flanking regions of the class 1 integron present in the animal strains detected in our study, as well as

evaluation of the clonal relatedness of isolates belonging to the same species, would better clarify if the In27 has been disseminated due to clonal expansion or by HGT events. Nonetheless, the fact that the same array was present in four different species is a strong indication that the class 1 integron dissemination is related with HGT between the isolates. In our study, PFGE and Southern blot hybridization showed that, at least in two of the isolates, *C. freundii* C16R385 and *E. cloacae* C2R379, the class 1 integron was located in a plasmid, which would facilitate the spread of the plasmid-borne integron by conjugation. In this work, natural transformation with these two donors did not produce transformants that acquired the class 1 integron.

The gene cassette *dfrA7* was, in our study, found in one isolate of *E. coli* collected from a food animal. Class 1 integrons with this cassette are also very common worldwide among animal isolates belonging to different species. Reports of this integron have been provided before, for example, in *S. enterica* serovar Typhimurium isolated from horses in The Netherlands (Vo et al., 2007b), in *E. coli* isolated from a dog in France (Skurnik et al., 2006), in *K. pneumoniae* collected in a brackish-water fish farm in Egypt (Ishida et al., 2010), in *E. coli* isolated in Ireland (Karczmarczyk et al., 2011), in *E. coli* collected from a herd animal in Australia (Barlow et al., 2009), and in *E. coli* and *S. enterica* subsp. *arizonae* isolated from flamingos in Japan (Sato et al., 2009). This integron is not restricted to animal bacteria, and has also been detected in *E. coli* and *K. pneumoniae* strains isolated from rivers in Turkey (Ozgumus et al., 2009); in *E. coli* collected from healthy humans in Australia (Bailey et al., 2010) and Korea (Kang et al., 2005); and in clinical samples of *A. baumannii* collected in South Africa (Segal et al., 2003), *A. pittii* from China (Xu et al., 2008a), *E. coli*, *K. pneumoniae*, *C. freundii*, *E. cloacae*, *Shigella* spp. and *Salmonella* sp. isolated in Central African Republic (Frank et al., 2007), *S. enterica* serovar Enteritidis collected in Spain (Rodriguez et al., 2011), and *S. enterica* collected in Uganda and South Africa (Krauland et al., 2009). Although this integron is widespread and HGT has been suggested (Krauland et al., 2009) and shown in some studies (Vo et al., 2007b; Rodriguez et al., 2011),

only one of the bacterial isolates from our study carried the class 1 integron with the *dfrA7* gene cassette. Determination of the genomic context of the integron would help to understand the factors contributing to the lack of distribution and low occurrence of this integron among the animal strains studied in our work. Limited sampling or sampling bias could also contribute to explain our observations. Our experimental work showed that the *dfrA7*-harbouring integron of *E. coli* strain C10R379 is located in the chromosome of the isolate, which suggests conjugation is not the major mechanism of HGT. Additionally, this isolate did not produce transformants in our natural transformation studies with trimethoprim as selection. These results also support a limited potential of dissemination of this integron among the bacterial isolates analysed.

The *E. fergusonii* AS041A2 isolate, collected from an owl, was in our study found to carry the gene cassette array *dfrA1-aadA1*. The same array has also been found in isolates collected from wild animals, both living freely or in captivity, such as in *E. coli* strains collected from seagulls in Portugal (Poeta et al., 2008), from black-headed gulls in Czech Republic (Dolejska et al., 2009), and isolated from zoo animals scarlet macaw, snowy owl and reticulated giraffe in Japan (Ahmed et al., 2007b), and in *E. coli* and *P. mirabilis* strains isolated from herring gulls in Italy (Gionechetti et al., 2008). Many of the reports of this class 1 integron in bacteria collected from wild animals are on birds (Stokes and Gillings, 2011), which strengthen the notion that birds can be a vehicle for integron dissemination. *E. coli* isolates collected from healthy dogs in Portugal (Costa et al., 2008); *E. coli*, *C. freundii* and *K. pneumoniae* collected in brackish-water fish farms located in Egypt (Ishida et al., 2010); *K. oxytoca* isolated from milk collected from cows with mastitis in Egypt (Ahmed and Shimamoto, 2011); *S. enterica* serovar Derby isolated from pork food product in China (Meng et al., 2011); *S. enterica* serovar Typhimurium isolated from horses in The Netherlands (Vo et al., 2007b); *E. coli* collected from chicken, pig, dog and horse in France (Skurnik et al., 2006); *E. coli* isolates with swine, cattle, poultry and dog origins, collected in Switzerland (Cocchi et al., 2007); and the Gram-positive *C. ammoniagenes* and *C. casei* isolated from poultry litter in USA

(Nandi et al., 2004); are a few examples of bacteria isolated from diverse animal sources, other than wild animals, containing the gene cassette array *dfrA1-aadA1*. As for the other gene cassette arrays found in the animal isolates in our study, also this array is found in clinical settings, including strains of *E. coli* isolated in Switzerland (Cocchi et al., 2007), *Proteus* sp. isolated in Australia (White et al., 2001), *E. hormaechei* subsp. *steigerwaltii* and *E. cloacae* cluster III collected in Poland (Mokracka et al., 2011); in human commensal isolates of *E. coli* collected in Switzerland (Cocchi et al., 2007) and Australia (Bailey et al., 2010); in *E. coli* and *Shigella sonnei* collected from one river in Portugal (Henriques et al., 2006) and from *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. vulgaris* isolated from Turkish rivers (Ozgumus et al., 2009); and in *E. coli* and *Aeromonas* sp. isolated in a wastewater treatment plant in Portugal (Moura et al., 2007). Although *E. fergusonii* has been associated with infections in humans and animals (Savini et al., 2008; Wragg et al., 2009), and isolated from a food product (Fegan et al., 2006), only a few studies report on class 1 integrons in this species (Barlow et al., 2009; Forgetta et al., 2012), which might indicate a low propensity of this species to undergo HGT or limited positive selection on integron-carrying members of the population. On the other hand, this assumption might be biased based on the fact that only few studies have been done on this species. In our work, natural transformation of *A. baylyi* BD413 with DNA from *E. fergusonii* AS041A2 did not produce any transformants. However, the integron-carrying recipient *A. baylyi* SD2 was transformed by this strain at similar transformation frequencies as other donor species. Therefore, the lack of transformants may be related to the limited potential for homologous recombination with the incoming DNA, rather than physical restriction of DNA uptake from *E. fergusonii*.

The *P. aeruginosa* strain Dog006M1, isolated from a dog, was in our study found to contain a class 1 integron with the gene cassette array *aadA6-orfD*. Among all the gene cassette arrays detected in our study, this is the least prevalent, according to the literature. The majority of the reports are on clinical isolates of *P. aeruginosa* isolates, which have been collected in France (Naas et

al., 1999), in United States (Borgianni et al., 2011), in Iran (Shahcheraghi et al., 2010), in China (Gu et al., 2007), in Mexico (Garza-Ramos et al., 2008), and in Australia and Uruguay (Martinez et al., 2012). A nucleotide sequence of the same *aadA6-orfD* array, detected in a *Pseudomonas* sp. strain collected from river water in Portugal, can also be found in GenBank (accession number JQ837991). A very recent study has reported for the first time the presence of the class 1 integron array *aadA6-orfD* in species other than *P. aeruginosa*; specifically, in isolates of *S. enterica* serovar Enteritidis collected from human and poultry in Iran (Firoozeh et al., 2012). These isolates, collected between November 2009 and June 2010, were recovered in the same region (Tehran) as a clinical isolate of *P. aeruginosa* carrying the same gene cassette array, collected in January 2009 (Shahcheraghi et al., 2010); the two arrays differ only by two nucleotides.

Class 1 integrons code for sulphonamide resistance (due to the *sull* gene). Ten out of the 11 animal bacteria isolates with a class 1 integron studied here contained a trimethoprim resistance gene. This observation may reflect the impact of the use of antibiotics in animal husbandry, as sulphonamides and trimethoprim are two of the most used antimicrobials in this field, usually in combination (Haller et al., 2002; Vo et al., 2007b). The use of these antibiotics may select for bacteria resistant to them or at least contribute to the maintenance of the integrons in the bacteria, which can subsequently be transferred to humans (Ferber, 2000; Smith et al., 2002), although there is no consensus on this issue (Phillips et al., 2004). From our results and from the scientific literature, we observed that the same gene cassette array is widespread among animal and human isolates.

2. Class 1 integron flanking regions

Our results on the analyses of the flanking regions of class 1 integrons are in accordance with the general knowledge that this class of integrons is associated with MGEs (Mazel, 2006). All the integrons in which the flanking regions were sequenced in our study were associated with transposons (Tn21-like, Tn1721-like, Tn5051-like), IS (IS26) or MITEs. In some cases, class 1 integrons integrated in a MGE were further inserted in plasmids (*S. enterica* serovar Typhimurium 490, *E. cloacae* C2R371 and *C. freundii* C16R385). These observations reflect the fact that class 1 integrons are embedded in MGEs with several layers of horizontal mobility, as often seen in the literature (Zienkiewicz et al., 2007; Doublet et al., 2009; Tato et al., 2010).

Our sequencing of the flanking as well as the conserved regions of the class 1 integrons of *A. baumannii* 65FFC and *A. bereziniae* 118FFC revealed that they were 100 % identical, only differing in the gene cassette array of the variable region. The nucleotide sequence beyond the variable region present in the class 1 integron of these clinical isolates, collected in 1998 in the University Hospitals of Coimbra, Portugal, was also the same as found in the *A. johnsonii* NFM2 isolated from a prawn in the ocean waters of Australia (Gillings et al., 2009a). The class 1 integron of these three *Acinetobacter* spp. isolates were flanked by two copies of a MITE-like structure with 439 bp, which had not been identified in other bacterial strains. Later in our study, the same MITE was detected flanking a class 1 integron present in clinical strains of *A. baumannii* collected in Brazil. Moreover, the regions flanking the conserved segments of the class 1 integron also appear to be identical to the other three *Acinetobacter* spp. isolates mentioned before. TSDs and interrupted genes, together with the observation that identical MITE-like structures are present in three different *Acinetobacter* species from three different continents, strongly suggest that MITEs may disseminate horizontally and act as a mobilisable vector for integron-resistance dissemination. It is interesting that this MITE has only been found in *Acinetobacter* species.

The evidence of this new structure (MITE-like) with identical nucleotide sequence in different *Acinetobacter* spp. isolates from different geographic location might indicate a broader dissemination of these MGEs among bacteria. Further studies may elucidate on the prevalence of these structures among the prokaryotic and clarify on detail their role on horizontal gene transfer.

II. Natural transformation of *A. baylyi*

1. Inter- and intraspecies transfer into integron free *A. baylyi* cells

Purified DNA substrates of *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490 were in our study found to transform *A. baylyi* BD413 cells at frequencies of 10^{-8} and 10^{-7} , respectively, over a 24 hrs time period. In total, eight transformants acquired a class 1 integron, four from each of these donors. The composition and order of gene cassettes in the donor bacteria was maintained in the transformant cells. Also, DNA lysates could transform *A. baylyi* BD413 cells, confirming that variability in DNA purity is of minor importance for HGT, as previously observed (Nielsen et al., 2000).

Our exposure of *A. baylyi* BD413 to DNA of the class 1 integron-containing *Salmonella* strain lead to horizontal transfer of its integron as part of transposon Tn21-like by transposition. The Tn21-like was found as single insert at different locations in three different examined transformant genomes. Exposure of *A. baylyi* BD413 to DNA of the class 1 integron containing *A. baumannii* strain lead to transformants with single chromosomal integrations of the integron either through DNA sequence similarity-based homologous recombination or randomly through genetic linkage to and movement of IS26 elements.

DNA sequencing of the flanking regions of the integrons in five initial transformants obtained after exposure to species-divergent donor DNA allowed

to verify their incorporation into the *A. baylyi* BD413 chromosome. The genomic locations of the integrons were also maintained in subsequent transformants ($n = 6$) obtained after exposure to DNA isolated from the initial transformants. Sequencing of the flanking regions of the transferred integrons revealed that not only the integron was acquired from the donor bacteria, but also additional DNA flanking the integrons. The length of the acquired, continual DNA fragments could be up to 23 000 bp long. The sequencing of the acquired DNA in five transformants obtained after exposure to DNA of different species, as well as the Southern hybridization of a total of eight transformants, revealed that integration had occurred into different regions of the *A. baylyi* genome.

Most interestingly, stable integration of the class 1 integron containing DNA in the *A. baylyi* chromosome had occurred by several mechanisms, depending on the flanking regions of the integrons. Transposition-based insertions were observed for integrons acquired from unrelated species. Specifically, DNA sequencing revealed that exposure to DNA substrates of *S. enterica* serovar Typhimurium 490 resulted in chromosomal integration of the acquired DNA due to the activity of transposase genes flanking the integrons; in this case, belonging to transposon Tn21-like. A class 1 integron is embedded in transposon Tn21 (Liebert et al., 1999), and many different gene cassette arrays have been reported for class 1 integrons present in Tn21-like transposons (Avila and de la Cruz, 1991). Therefore, the horizontal dissemination of this transposon is a major contributor to the dissemination of class 1 integrons. Conjugation of the plasmids where this, or other, Tns are often inserted, allows the propagation of Tn21-like transposon and the embedded class 1 integron (Liebert et al., 1999; Labbate et al., 2008). Here, we have experimentally demonstrated that natural transformation can facilitate interspecies movement of this transposon. A previous study of Partridge et al. (Partridge et al., 2002) also indicated transposition as a possible mechanism of integron movement. However, no experimental transposition tests were performed in that study. For a related species (*A. baumannii* DNA donor), homologous recombination-based insertion was observed, as expected (Ray et al., 2009). However, in this latter exposure scenario, also site-specific recombination due to IS26-associated

transposition was observed in our study. Similarly to Tn21-like, IS26 elements are often associated with antibiotic resistance genes and presumed active in *Acinetobacter* sp. (Post et al., 2010). There are several reports of IS26-associated integrons (Miriagou et al., 2005; Jeoung et al., 2009), and transposition of IS26-composite transposons has been suggested (Doublet et al., 2009). The presence in the recipient cell of nonfunctional IS26 could also allow the capture of additional resistance genes by homologous recombination-mediated DNA integration, if a homologous IS26 region is present in the donor DNA fragments. Such recombination scenario could explain, for example, the dissemination of the chromosomally-located integron-IS26 in *A. baumannii* in South Korea (Han et al., 2008; Jeoung et al., 2009).

Although interspecies transformation frequencies of integrons are low, we observed that intraspecies transfer, between *A. baylyi* cells with and without integrons, occurs at higher frequency. Therefore, integrons can be quickly spread between cells belonging to the same species. In this situation, transfer of integrons likely occurs by homologous recombination.

Annealing of complementary DNA strands in the cytoplasm is considered necessary for re-circularization, and hence stable uptake of plasmids in competent bacterial species (Saunders and Guild, 1981). Drawing on this general observation, we hypothesized that annealing of other ssDNA taken up into the cytoplasm of competent bacterial cells would allow transient expression of genes present on such linear fragments. For instance, expression of recombinase genes present in mobile genetic elements in these dsDNA fragments could lead to integration of the elements into the host chromosome; this was probably what happened to allow expression of the transposases of Tn21-like and IS26-composite transposons, with consequent transposition. To what extent natural transformation of bacteria can facilitate intergenomic mobility of a wider range of mobile genetic elements being part of species-foreign, linear DNA fragments, can, and should, be tested in further studies.

The biological properties of natural transformation as a mechanism that can facilitate DNA translocation over bacterial membranes and site-specific

recombination (e.g. active transposition events of transposons) in bacterial genomes could be further explored. For instance, model systems can be developed using small DNA fragments with site-specific recombination functions as donor DNAs. Moreover, possible interactions between recombinases encoded by the incoming DNA and the host itself should be investigated. For example, the *A. baylyi* ADP1 strain harbours two prophages, a number of transposases, and six copies of IS1236, with two of them flanking Tn5613 (Barbe et al., 2004). Different species of competent bacteria could also be used as recipients in natural transformation assays, to test whether the phenomenon of transposition after uptake of DNA by natural transformation is a generalized mechanism among competent bacteria or if it is limited to *A. baylyi*.

MITEs are expected to promote the transposition of flanked DNA. However transfer of the class 1 integron of *A. baumannii* 65FFC by natural transformation of *A. baylyi* BD413 was not observed in our study. This result suggested that the transposase required for movement of the MITEs is not present or expressed in any of the strains, or that the transformation frequency of class 1 integron MITE-facilitated transposition is below the detection limit ($< 10^{-9}$). In order to test if an active transposase would promote transposition of MITEs, the transposition module of Tn21-like from *S. enterica* serovar Typhimurium 490 was cloned into *A. baylyi* BD413, resulting in the strain *A. baylyi* SD12. However, transformation assays with this strain as recipient also failed to produce transformants with acquired class 1 integron. Several possible causes can be related with this failure. First of all, in order to catalyse transposition, the transposase must recognize the IRs of the mobile element (Lupski, 1987). It is possible that the TnpA present in *A. baylyi* SD12 does not specifically recognize the ends of the MITEs, which are much shorter than the IRs of Tn21, and, therefore, is not able to promote their movement. In future studies, different transposases, belonging to different Tn groups and IS families, can be introduced into the *A. baylyi* BD413 genome, and the resulting transformants can be used as recipients in transformation assays and tested for the ability to promote MITEs transposition. Another limitation on the activity of the

transposition module present in *A. baylyi* SD12 might be related with the absence of the ribosomal binding site and the promoter of the *tnpM* gene; as it has been suggested that this gene enhances transposition (Liebert et al., 1999). The ribosomal binding site and the promoter of the *tnpM* gene are within the integron and were not included in the amplified DNA containing the transposition module, to exclude the presence of homologous regions that could allow homologous recombination to occur. The introduction of the transposition module including the missing sequences in a *recA*-deficient strain of *A. baylyi* BD413, which could be used as recipient, would help to clarify this point.

Due to the fact that the use of low concentrations of β -lactams in transformation selection can result in a high number of transformants, selection in the transformation assays with *A. baylyi* SD12 as recipient and *A. baumannii* 65FFC as donor was done with high concentration of cefotaxime (50 μ g/ml). However, it might have been a too high selection pressure for the growth and establishment of newly formed transformants. Therefore, mobilization of a class 1 integron promoted by the flanking MITEs might be tested with different donors, which will allow the use of a different selection regime.

The majority of the DNA substrates exposed to *A. baylyi* BD413 cells produced transformants in our study, independently of the genetic relatedness between both the donor and the recipient, as determined by phenotypic screening on antibiotic-containing growth media. However, subsequent genotypic screening (PCR) showed that only some of the transformants with changed antibiotic resistance profiles had acquired entire class 1 integrons. The observation of variable changes in susceptibility patterns, after exposure to various sources of bacterial DNA, without detectable DNA acquisitions, suggests a broad potential of and the presence of a yet not described range of transferable resistance determinants in bacteria, of unknown mechanistic and genetic nature. It is known that transfer of small DNA fragments (< 1000 bp) can be associated with the formation of mosaic genes, leading to changes in the antimicrobial susceptibility pattern of bacteria; and that mosaic genes are mainly the result of transformation events (Maiden, 1998). In most studies published so far, the

recombination events resulting in mosaic genes occurred between isolates belonging to the same species or same genera (Lujan et al., 1991; Radstrom et al., 1992). In contrast, we noted changes in susceptibility profiles resulting from natural transformation of *A. baylyi* with both related and unrelated donor species and genera (*A. baumannii*, *P. aeruginosa*, *S. enterica* serovar Rissen and serovar Typhimurium). Our study suggests that exposure of competent bacteria to heterologous sources of DNA may produce complex changes in resistance profiles, not necessarily predictable from the known resistance genes present in a given donor isolate. Broader studies are necessary to reveal the exact nature of such changes. Recent studies reported transfer of numerous chromosomal polymorphisms between *Haemophilus influenza* (Mell et al., 2011) and *S. pneumoniae* and *Streptococcus mitis* genomes (Sauerbier et al., 2012) by natural transformation.

Gomez and Neyfakh detected some genes involved in intrinsic antibiotic resistance in *A. baylyi* (Gomez and Neyfakh, 2006). A screen of these genes was done with 12 different antibiotics, which did not include sulphonamides. Some of the transformants obtained in our study showed reduced susceptibility to this group of antibiotics, for which mosaic genes are also known to be formed (Maiden, 1998). A similar study to the one done by Gomez and Neyfakh (Gomez and Neyfakh, 2006) can be performed in order to attempt the identification of the genes involved in intrinsic antibiotic resistance to sulphonamides in *A. baylyi*. If such genes are present, the sequence of those genes in transformants with reduced susceptibility to sulphonamides might identify newly formed mosaic genes.

2. Interspecies transfer into integron-containing *A. baylyi* cells

Natural transformation of the class 1 integron-carrying *A. baylyi* SD2 recipient with DNA isolated from different species of donor bacteria gave comparable transformation frequencies to those obtained with DNA of the same species.

Thus, interspecies acquisition of class 1 integrons that take place at low initial frequencies can be followed by high frequency interspecies dissemination.

All tested transformants acquired the gene cassette composition of the integron of the donor bacterium, rather than acquiring additional new gene cassettes in the existing integron. This result suggests that homologous recombination replaces gene cassettes through gene replacement caused by crossover junctions forming at the conserved end segments of the class 1 integron, rather than site-specific recombination of gene cassettes promoted by the *intI1* integrase. A role of the *intI1* integrase was not observed in the acquisition of new gene cassettes, when exchange occurs between two class 1 integron-carrying bacterial isolates.

In the transformation experiments performed with recipient cells containing a class 1 integron with the integrase gene inactivated (strain KOI), transformation frequencies were the same as with the recipient with an active integrase (strain SD2). Transformation with recipient cells containing a class 1 integron but with the *recA* gene inactivated (strain RAM), resulted in four orders of magnitude reduction in transformation frequencies. These two results confirmed the role of homologous recombination (Schmitt et al., 1995; Ray et al., 2009) in the replacement of integrons and gene cassettes in class 1 integron-containing recipient bacteria. Partridge *et al.* (Partridge et al., 2002) also suggested replacement of a gene cassette array in *P. aeruginosa* to have occurred by homologous recombination. Here we provide experimental evidence that homologous recombination can efficiently replace gene cassette arrays. Mobile genetic elements thus provide sufficient DNA similarity for homologous recombination to occur between otherwise unrelated bacterial species. The presence of DNA similarity between species as a result of the presence of MGEs can therefore likely contribute to the formation and spread of chromosomally-located complex mosaic regions, often formed by several resistance genes and mobile elements (Stokes and Gillings, 2011).

As mentioned before, *A. baylyi* does not have a LexA orthologue (Hare et al., 2006). Therefore, the integrase genes present in this species may be

derepressed or regulated by other unknown factors. Expression of the *intI1* integrase gene in the recipient *A. baylyi* SD2 was not found in the RT-PCR assay. The absence of detected rearrangements of gene cassettes in our study might be explained by the absence (or low) expression of the integrase in the recipient *A. baylyi* SD2, and by the fact that the homologous recombination events occur much more frequently than integrase-catalysed cassette recombination events. Therefore, rare rearrangements of gene cassettes may have occurred in our model system but would not be detectable in the number of transformants that were genetically characterized. The recent work published by Baharoglu et al. (Baharoglu et al., 2010) on conjugative induction of SOS and its ability to trigger cassette recombination showed that cassette rearrangements occurred at very low frequencies (e.g. 10^{-8}) in *V. cholerae*. The relative low frequency of integrase-facilitated cassettes recruitment and rearrangements, compared with the frequency of homologous recombination, can also help explain why some gene cassette arrays are common and shared between different bacterial species (Partridge et al., 2009).

III. Fitness cost of acquired class 1 integrons embedded in MGEs

Intervention policies aimed at combating antibiotic resistance often rely on the assumption that resistance genes are costly and will be lost from pathogenic bacterial populations when antibiotic pressure is removed (Johnsen et al., 2009). However, our relative fitness measurements suggest that the acquired class 1 integrons and the resulting changes in resistance profiles did not cause major fitness reductions of transformants, or if they did, compensatory mutations may have ameliorated such cost. The selection of CFUs in our fitness studies was based on the resistance trait conferred only by the class 1 integron. It is, however, noted that the fitness measurements performed in our study represented the combined effect of all transferred DNA segments, as well as

effects on the transformant genome due to insertion effects. The specific fitness effects of the variable amount of additional genes acquired by the transformant were not determined in our study. However, it is expected that such DNA will affect the fitness of the host, with both positive and negative contributions. A recent study determined the fitness cost of horizontally transferred class 1 integrons alone, free of associated transposable elements, and indicated a negative fitness effect of newly acquired integrons (Starikova et al., 2012). It is speculated that traits conferred by the MGEs associated with integrons may alleviate the negative fitness costs of integrons in their new hosts.

Future studies can determine the fitness effect of class 1 integrons with different gene cassette arrays, as well as larger DNA segments, such as transposons, inserted into the neutral locus *lipAB* of the chromosome of *A. baylyi* BD413. This will allow a more precise determination of the correlation between fitness changes and the presence of multiple resistance gene/vector combinations. In cases of rapid host adaptation, efforts should be done to identify the molecular changes leading to compensatory evolution. A previous study (Enne et al., 2005) assessed the fitness cost of antibiotic resistance genes encoded by one plasmid, two transposons and one mutation in one essential gene, both *in vitro* and *in vivo*; the antibiotic resistances were introduced into the host by conjugation. The authors concluded that low or no cost was imposed by the antibiotic resistance elements, and that compensatory evolution was seen over the time course of the experiments (Enne et al., 2005). The study by Starikova *et al.* showed that the fitness cost imposed by acquisition of class 1 integrons by natural transformation, could be restored due to mutations in the class 1 integrase gene (Starikova et al., 2012).

IV. Main conclusions

The majority of the class 1 integrons described so far contributes to the dissemination of antimicrobial resistance genes (in addition to other gene

cassettes with unknown function), and our study provides new information on the dissemination of antimicrobial resistance between genetic divergent bacteria beyond plasmid-based conjugation. Such natural transformation events, even if occurring at low frequencies, can be of importance as they result in the initial acquisition of a class 1 integron-carrying MGE. The element can subsequently serve as a new genetic platform for the efficient capture or exchange of additional resistance gene cassettes.

The amount of species naturally competent may be much wider than what it is known so far (Johnsborg et al., 2007). Furthermore, cultivation-based selection of transformants on antibiotic-containing agar plates underestimates transformation frequencies, compared with frequencies achieved in situ (Rizzi et al., 2008).

Natural transformation could, for instance, be responsible for the dissemination of chromosomal class 1 integrons in *P. aeruginosa* (Martinez et al., 2012) or *S. enterica* serovar Typhimurium (Vo et al., 2007b). Recently, the acquisition of (synthetic) gene cassettes by natural transformation in a *Pseudomonas* species (Gestal et al., 2011) was also shown; indicating that homology-independent DNA acquisitions by natural transformation might be a more general phenomenon. It is also worth noting that chromosomal SIs are mainly found in *Vibrio* species, which are naturally transformable (Mazel et al., 1998; Johnsborg et al., 2007).

Our results indicate that natural transformation of DNA fragments is not necessarily limited by requirements of high DNA sequence similarity for stable integration to occur, and that genetically unrelated bacteria can exchange genetic material, such as class 1 integron-containing transposons, through natural transformation, provided the transferred DNA fragments encode functions for recombination. As observed in our studies, the genetic signature of events of natural transformation can be limited to the site-specific insertions of transposons. However, the observation of transposons or insertion sequences in a given bacterial genome is usually not causally attributed to events of natural transformation. Thus, retrospective DNA sequence analysis would not have

considered natural transformation as the causal mechanism until now. This study suggests a significant potential for the interspecies spread of mobile genetic elements such as Tns and ISs through natural transformation. Hence, presenting a new pathway for horizontal dissemination of antimicrobial resistance determinants present in class 1 integrons embedded in transposons. The potential for dissemination of class 1 integrons should be seen as the product of the factors governing the linkage of integrons to MGEs, the supply of MGEs into a given bacterial population, their heritable stability in new bacterial hosts, and the larger opportunity for MGE carrying hosts to co-exist with or outcompete non-carrying members of the larger population. Thus, considerations of genetic linkage, dissemination rates and absolute and relative host fitness effects are essential to understand the fate of integrons. It is further important to recognize that fitness will be a variable depending on both host and environmental factors; including continual changes in the genetic composition of the host and integron-containing MGE. Further complicating factors are bacterial population structure and transmission dynamics, spatial and-temporal variability in transfer rates (exposure dependency), directional selection (e.g. temporal antibiotic usage), and randomness in most processes including host survival and dissemination, leading to non-uniform outcomes.

Many environmental factors can be controlled in experimental laboratory model systems leading to the opportunity to conduct quantitative descriptions of integrons and their dissemination characteristics. However, in clinical and other environments, variability and random fluctuations in the governing factors considered above rarely leads to opportunities for a precise quantitative understanding of the transfer dynamics of integrons and the factors that determine current dissemination patterns.

The knowledge of the structures and the mechanisms that facilitate movement of integrons with resistance gene cassettes is an important step to develop effective measures to control antibiotic resistance and to unravel the molecular mechanisms behind HGT leading to rapid adaptation to pharmaceuticals. Even with the ban of antimicrobials in some settings, the removal of antimicrobial resistance does not seem likely, and finding ways of reducing selection of

resistance or blocking the further spread of resistance genes by HGT might be the future direction we should pursue. For instance, a recent study demonstrated that copper alloys prevent HGT events (Warnes et al., 2012). More studies should be done in order to unravel the mechanisms involved in the spread of antimicrobial resistance genes, as well as finding ways of preventing such mechanisms.

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