Natural Transformation Facilitates Transfer of Transposons, Integrons and Gene Cassettes between Bacterial Species

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Abstract

We have investigated to what extent natural transformation acting on free DNA substrates can facilitate 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 integron-carrying strains of the genera Acinetobacter, Citrobacter, Enterobacter, Escherichia, Pseudomonas, and Salmonella to determine the nature and frequency of transfer. Exposure to the various DNA sources resulted in acquisition of antibiotic resistance traits as well as entire integrons and transposons, over a 24 h exposure period. DNA incorporation was not solely dependent on integrase functions or the genetic relatedness between species. DNA sequence analyses revealed that several mechanisms facilitated stable integration in the recipient genome depending on the nature of the donor DNA; homologous or heterologous recombination and various types of transposition (Tn21-like and IS26-like). Both donor strains and transformed isolates were extensively characterized by antimicrobial susceptibility testing, integron- and cassette-specific PCRs, DNA sequencing, pulsed field gel electrophoreses (PFGE), Southern blot hybridizations, and by re-transformation assays. Two transformant strains were also 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. Moreover, natural transformation of integron harboring populations of competent bacteria revealed that interspecies exchange of gene cassettes can be highly efficient, and independent on genetic relatedness between donor and recipient. In conclusion, natural transformation provides a much broader capacity for horizontal acquisitions of genetic elements and hence, resistance traits from divergent species than previously assumed.

Introduction

The acquisition and dissemination of antibiotic resistance in Gram-negative bacteria is frequently facilitated by integrons [1–3]. Integrons contain genetic determinants for site-specific recombination and promoters driving the expression of gene cassettes [4]; the integrase (IntI) encoded by the integron facilitates site-specific acquisitions and excisions of gene cassettes within the integron [5–8]. The gene cassettes often encode antibiotic resistance, however cassettes conferring other metabolic functions to bacteria have also been described [9–11]. Integrons and integrases have been found to be present in approximately 9 to 17% of the sequenced bacterial genomes [1,12]. Class 1 integrons are the most widely disseminated type in commensals and pathogens of human and animal origins [13–18], and have also been found in soil and in aquatic ecosystems [19–23]. This class of integrons is characterized by two conserved regions, the 5’ conserved segment (5’-CS), which includes the integrase gene (intI1), the adjacent recombination site (attI) and the promoter (Pc), and the 3’ conserved segment (3’-CS), which contains the qacEA1 gene (encoding an incomplete version of a quaternary ammonium compound resistance), the sulI (encoding resistance to sulfonamides) and the aof5 [2,24].

Highly similar class 1 integrons have been found in both Gram-negative and -positive bacteria [25,26] and integrons with the same composition and organization have been found in unrelated bacterial species and strains in geographically distinct areas [27–30]. The exceptional broad potential of integrons to disseminate among pathogenic bacterial strains and species is remarkable because they only harbor functions for genomic integration and excision and do not encode functions that enable horizontal transfer between bacterial cells. Class 1 integrons are often present in plasmids, transposons and insertion sequences [12,31,32], and their dissemination is considered to depend on horizontal mobility of the genetic element or the genetic region they reside drawing on...
Natural Transformation of Genetic Elements

Author Summary
Genetic elements, such as transposons and integrons, frequently carry antimicrobial resistance determinants and can be found widely disseminated among 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. We have tested if natural transformation can facilitate transfer of transposons and class 1 integrons between bacterial species. We here provide experimental evidence showing that natural transformation can be a general mechanism for dissemination of genetic elements that by themselves do not encode interspecies transfer functions (e.g. transposons, insertion sequences). We demonstrate that antibiotic resistance determinants present in such genetic elements can spread by natural transformation between species of clinical interest. We show by quantitative data that interspecies exchange of resistance gene cassettes is highly efficient among integron-containing strains and species. Our study also provides a plausible explanation for how sequence-conserved integrons can become distributed among bacterial species.

Materials and Methods
Bacterial strains and cultures
The naturally competent soil bacterium A. baylyi BD413 (spontaneous rifampicin resistant mutant) [47] and close derivatives (this work) were used as recipients. A highly similar strain (ADP1) has been sequenced (acc. no. CR543861) [44]; only few differences between the two laboratory strains are expected [48]. The integron-carrying bacteria were A. baumannii 064, A. baumannii 65FFC, P. aeruginosa K34-73, K. pneumoniae K66-45, both carrying the NDM-1 metallo-β-lactamase among other resistance genes, and P. aeruginosa K34-73, carrying the VIM-4 metallo-β-lactamase (Table 1). The NDM-1 genes of both E. coli K71-77 and K. pneumoniae K66-45 are plasmid-encoded; P. aeruginosa K34-73 carries a class 1 integron with four gene cassettes (blaVIM-4, arr-7, qacE4, blqSE6) in the chromosome. The transformants yielded from exposure to DNA from these 3 latter strains were only characterized at the phenotypic level.

Some of the transformants of A. baylyi that were confirmed to have taken up the integron from the heterologous donor sources were also used as a source of donor DNA in subsequent transformation experiments (i.e. isolates SD1, SD2, SD3, SD4, SD5 and SD6; see Table 1). One of the integron-carrying transformants of A. baylyi (isolate SD2) was also used as a recipient bacterium in subsequent transformation assays (see Table 1).

Other derivatives of the A. baylyi BD413 strain employed as recipients (Table 1) were SD9 (a ΔrecBCD ΔrecD double mutant constructed as described by Harms and Wackernagel [49]) and the SD2 derivatives KOI (intI1::cat) and RAM (rec::cat) constructed as follows: an internal segment (724 bp) from the intI1 gene of A. baumannii 064 was PCR-amplified using the primers intI1-f (5′-AGCTTACGAA CGGAACAGGC-3′) and INCINTF (5′-TGATGCGCTGC TTGTCTTACG-3′) and Phusion DNA polymerase (Finnzymes, Finland), according to the manufacturer's instructions, and inserted into the Smal site of pACYC177, resulting in pACYC177-int36. Next, a 1077 bp segment covering the cat (chloramphenicol resistance) gene from pACYC184 was cloned into the singular XmnI-linearized and used to inactivate the recA gene of A. baumannii 064. The transformants yielded from exposure to DNA from these 3 latter strains were only characterized at the phenotypic level.

Both the donor strains and the transformant isolates were characterized by antibiotic resistance profiling, targeted PCR, DNA sequencing by extensive primer walking, genome sequencing of two transformants, pulse-field gel-electrophoresis (PFGE) and Southern blot hybridization. A pairwise growth competition assay was undertaken to determine the impact of the acquired integrons on relative fitness. The integron-carrying transformants of A. baylyi were also used in subsequent transformation assays to confirm the nature of the initial interspecies gene transfer and determine further intraspecies transfer frequencies.

Both transduction and conjugal transfer processes [33-35]. However, horizontal movement of class 1 integrons genetically linked to non-conjugative elements [36] or incomplete mobile genetic elements and transposons [20,32,37] remains to be explained. 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 [38], the acquisition and integration of synthetic gene cassettes by natural transformation [39] and transposition of the integron In33 [40,41]. Horizontal transfer of non-conjugative transposons seems to rely on linkage to conjugative elements or on transduction by bacteriophages [42,43].
Table 1. Summary of the strains used in this study.

| Strain                  | Origin         | Plasmid Status | Integron size (bp) | Gene cassettes                          | Relevant phenotype/trait | Reference                       |
|-------------------------|----------------|----------------|-------------------|----------------------------------------|--------------------------|--------------------------------|
| **DNA donors:**         |                |                |                   |                                        |                          |                                 |
| Acinetobacter baumannii | Clinical       | Yes            | 762               | aadB                                   | K<sup>a</sup>             | This study                     |
| A. baumannii 064        | Clinical       | No             | 1000              | bla<sub>RAM</sub>S                     | CTX<sup>b</sup>           | [28]                           |
| A. baumannii 65FFC      | Transformant   | No             | 2000              | bla<sub>OXA</sub>30+aadA1              | Sc<sup>m</sup>            | This study                     |
| A. baumannii 064        | Transformant   | No             | 762               | aadB                                   | K<sup>a</sup>             | This study                     |
| A. baumannii 065FFC     | Transformant   | No             | 2000              | bla<sub>OXA</sub>30+aadA1              | Sc<sup>m</sup>            | This study                     |
| A. baumannii 064        | Transformant   | No             | 1000              | bla<sub>RAM</sub>S                     | CTX<sup>b</sup>           | [28]                           |
| A. baumannii 65FFC      | Transformant   | No             | 1912              | dfrA12+aadA2                           | Sc<sup>m</sup>            | This study                     |
| A. baumannii 064        | Transformant   | No             | 3000              | aacA4+bla<sub>PSE</sub>+aadA2         | Sc<sup>m</sup>            | This study                     |
| A. baumannii 064        | Transformant   | No             | 3000              | aacA4+bla<sub>PSE</sub>+aadA2         | Sc<sup>m</sup>; intI<sub>1</sub>cat | This study                     |
| Citrobacter freundii    | Rabbit         | Yes            | 1913              | dfrA12+orfF+aadA2                      | Sc<sup>m</sup>            | Domingues et al., unpublished |
| Enterobacter cloacae    | Rabbit         | Yes            | 1913              | dfrA12+orfF+aadA2                      | Sc<sup>m</sup>            | Domingues et al., unpublished |
| Escherichia coli C10R379| Rabbit        | No             | 769               | dfrA7                                  | W<sup>c</sup>             | Domingues et al., unpublished |
| Escherichia coli K71-77* | Clinical     | Yes            | n.d.              | -----                                  | CN<sup>a</sup>            | [106]                          |
| Escherichia fergusonii  | Owl            | Yes            | 1594              | dfrA1+aadA1                            | W<sup>c</sup>             | Domingues et al., unpublished |
| Klebsiella pneumoniae   | Clinical       | Yes            | n.d.              | -----                                  | CN<sup>a</sup>            | [106]                          |
| Pseudomonas aeruginosa  | Clinical       | No             | 3604              | bla<sub>OXA</sub>30+aadA1              | CN<sup>a</sup>            | [107]                          |
| P. aeruginosa SM        | Clinical       | No             | 3000              | aacA4+bla<sub>PSE</sub>+aadA2         | Sc<sup>m</sup>            | [67]                           |
| Salmonella enterica     | Fresh pork sausage | Yes     | 1912              | dfrA12+aadA2                           | Sc<sup>m</sup>            | This study                     |
| S. enterica serovar Rissen 486 | Pork hamburger | Yes        | 2000              | bla<sub>OXA</sub>30+aadA1              | Sc<sup>m</sup>            | Da Silva, unpublished          |
| **Recipients:**         |                |                |                   |                                        |                          |                                 |
| Acinetobacter baileyi   | Soil           | No             | No                | -----                                  | R<sup>a</sup>             | [47,108]                       |
| A. baileyi SD2          | Transformant   | No             | 762               | aadB                                   | K<sup>a</sup>             | This study                     |
| A. baileyi KOI          | Transformant   | No             | 762               | aadB                                   | K<sup>a</sup>; intI<sub>1</sub>cat | This study                     |
| A. baileyi RAM          | Transformant   | No             | 762               | aadB                                   | K<sup>a</sup>; recA<sub>C</sub>cat | This study                     |
| A. baileyi SD9          | Transformant   | No             | No                | -----                                  | ΔrecB<sub>CD</sub> Δsbc<sub>CD</sub> | This study                     |
| A. baileyi KOC4         | Transformant   | No             | No                | -----                                  | W<sup>c</sup>, ΔcomFECBΔdhfr | This study                     |
| **Genome sequenced:**   |                |                |                   |                                        |                          |                                 |
| A. baileyi (AbI13)      | Transformant   | No             | 762               | aadB                                   | K<sup>a</sup>             | This study                     |
| A. baileyi (StI3)       | Transformant   | No             | 2000              | bla<sub>OXA</sub>30+aadA1              | Sc<sup>m</sup>            | This study                     |

<sup>a</sup>Obtained with 5'-CS and 3'-CS primers [24].

<sup>b</sup>The resistance trait was used for selection in the natural transformation assays.

<sup>c</sup>Transformants obtained after exposure to these sources of DNA were only tested at the phenotypic level.

<sup>d</sup>Obtained by natural transformation of A. baileyi BD413 with purified DNA from A. baumannii 064. Integron acquisition was shown first by PCR, which explains the antimicrobial susceptibility profile of the tested antibiotics. The PFGE profile obtained with restriction digestion using the enzyme I-CeuI revealed unexpected bands that co-hybridized with the probe for intI<sub>1</sub> gene in Southern blot.

<sup>e</sup>Obtained by natural transformation of A. baileyi BD413 with purified DNA from S. enterica serovar Typhimurium 490. Integron acquisition was shown first by PCR, which explains the antimicrobial susceptibility profile of the tested antibiotics. Initial sequencing of the integron flanking regions, by primer walking, showed that the acquired fragment was large; suggestive of the possible acquisition of a plasmid (excluded after sequencing).

<sup>f</sup>Resistance: CN – gentamicin, CTX – cefotaxime, K – kanamycin, R – rifampicin, SC – spectinomycin, W – trimethoprim.

n.d. - not determined.

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with antibiotics according to their phenotype: ampicillin (AM; 25 μg/ml), cefotaxime (CTX; 10 μg/ml), chloramphenicol (Cl; 5 or 10 μg/ml), gentamicin (CN; 10 μg/ml), kanamycin (K; 10 μg/ml), R (25 μg/ml) spectinomycin (SC; 10 μg/ml) and trimethoprim (W; 250 μg/ml). A. baylyi cells and transformants were grown at 30°C, and the different donor bacteria at 37°C.

DNA extraction
Genomic DNA used in the transformation assays (10 μg) was isolated from bacterial cultures using anion exchange columns (Qiagen, Germany) columns according to the manufacturer’s protocol and resuspended in EB buffer, pH 8.5 (Qiagen, Germany). Plasmid DNA was isolated using a Plasmid Miniprep Kit (Qiagen, Germany). The DNA concentration was measured with a UV/VIS spectrophotometer (6405 Spectrophotometer, Jenway, England) or a Nanodrop ND-1000 (Nanodrop Technologies, USA).

For the preparation of supernatants (lysat) of the heat-treated bacterial cell suspensions, 5 ml overnight cultures of the bacteria were centrifuged at 20,000×g for 5 min and resuspended in water (50 μl), followed by heat treatment at 80°C for 15 min. The raw lysate was centrifuged, and the supernatant containing DNA was collected [51]. The lack of viable cells was confirmed by streaking aliquots on LB plates.

Natural transformation assays
The recipient cells were prepared, and experiments were performed on nitrocellulose filters placed on agar-surfaces, as previously described [52,53]. Each transformation assay was repeated between two and ten times (each assay was done in triplicate). Transformation assays were done with: i) A. baylyi BD413 as recipient and DNA extracted from various wild-type integron-carrying species; ii) with A. baylyi BD413 as recipient and DNA extracted from integron-carrying A. baylyi transformants; iii) with integron-carrying A. baylyi transformant SD2 as recipient and DNA extracted from various wild-type integron-carrying species; iv) with an integrase deletion carrying A. baylyi transformant (KO1) as recipient and DNA extracted from various wild-type integron-carrying species; v) with an integron-carrying A. baylyi recD deletion (RAM) recipient and DNA extracted from various wild-type integron-carrying species; vi) with the double mutant strain A. baylyi SD9 (ΔrecBCD ΔtheCD) as recipient; vii) and with competence mutant strain A. baylyi KO4C (ΔcomFECB) as a recipient. Selection of the transformants was done with different antibiotics and concentrations (see above in “bacterial strains and culture” and Table 1), according to the known or established resistance levels of the donors as determined by resistance typing and MIC determination.

A positive control was included to verify recipient cell competence and reproducible experimental conditions (transformation of A. baylyi BD413 by DNA from A. baylyi KTG which contains a chromosomally located npilI [kanamycin resistance] gene [47,53]). As negative control recipient cells were streaked on LBR plus selective antibiotic without addition of the donor DNA. The transformation frequencies were calculated for each transformation assay and are given as the number of transformants divided by the number of viable recipient cells.

Antimicrobial susceptibility tests
Antimicrobial susceptibility of donor, recipient and transformant bacteria was assessed by the disk diffusion method and determination of the minimal inhibitory concentrations (MICs). Both methods were performed according to the CLSI (Clinical Laboratory Standards Institute) guidelines [54], using Mueller-Hinton II (Fluka, BioChemika, Switzerland or Scharlau Chemie S.A., Spain) or PDM (AB Biodisk, Sweden) agar plates. The antibiotic susceptibilities tested for the various donor and transformant bacteria were determined according to the gene cassettes present. The antimicrobial disks used (Oxoid, England or AB Biodisk, Sweden) were: amikacin (30 μg), amoxicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), cefotaxime (30 μg), cefazidime (30 μg), chloramphenicol (5 μg), compound sulphonamides (300 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), meropenem (10 μg), netilmicin (30 μg), rifampicin (5 μg), spectinomycin (100 μg), sulfadiazine (250 μg), streptomycin (10 μg), sulfamethoxazole/trimethoprim (25 μg), trimethoprim (5 μg), tobramycin (10 μg). When the antimicrobial susceptibility profile of transformants had changed, the E-test method (AB Biodisk, Sweden) was used to quantify the MICs. MICs were also determined prior to the experiments for both recipient and donor bacteria, to determine the appropriate concentration of antibiotics to be used for transformant selection. The following E-tests were used: ampicillin, cefotaxime, cefazidime, gentamicin, kanamycin, spectinomycin, sulphonamethoxazole, and tobramycin.

PCR-based detection of integrons, gene cassettes, insertion sequences and other resistance determinants
The presence of class 1 integrons in the donor, recipient and transformant bacteria was assessed by PCR. PCR assays were set up in two different mixtures: 25 μl final volume of 22.5 μl PCR SuperMix (Invitrogen, Alligene, Portugal), 0.75 μl of each primer 10 μM and 1 μl (approx. 10 ng DNA) of lysate DNA; or 50 μl final volume using 22.5 μl of the 2× PCR MasterMix Dynazyme II from Finnzymes (Finzymes, Finland), 0.75 μl of each primer 10 μM, 25 μl of sterile water and 1 μl (approx. 10 ng DNA) of lysate DNA. PCR amplification was performed by a T-personal (Biometra, Goettingen, Germany), a MJ Mini (BIO-RAD, Portugal) or a PTC-200 (BIO-RAD, Norway) thermal cycler. Class 1 integrons were detected with a set of primers specific for the 5′-CS and the 3′-CS regions [24] or for the conserved regions of the class 1 integrase gene, IntI1 [55]. 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, 1 min), and a single final extension of 16 min at 72°C for detection of integrons [28] or was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 45 s, followed by a final extension time of 10 min at 72°C [55], for amplification of the integrase gene.

Rearrangements of gene cassettes during integron transfer and transformant cultivation were tested by PCR combining one primer for the 5′-CS region and one primer that binds in the distal gene cassette in the integron of the donor bacterium.

For some transformants, obtained with strains SD2, KO1 and RAM as recipients, the presence of the gene cassette aadBl was determined by PCR with primers AADB1 (5′-AGCCAAGCAG-GATGATATTG-3′) and AADB2 (5′-CGAAGACCT-CAACCTTTTC-3′) for 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C, followed by 10 min at 72°C.

Transformants with reduced susceptibility to some antibiotics but without a positive detection of the entire integron by PCR, were screened for the acquisition of different resistance determinants. PCRs were performed that targeted the gene cassettes present in the variable region of the integrons and the genes present in the 3′-CS of the integron of the corresponding donor bacteria, with specific primers for each cassette. The presence of the insertion sequence ISAbOl [56] was screened for by PCR in the
recipient *A. baylyi*, in *A. baumannii* donors and in transformants with reduced susceptibility to ampicillin. For all PCR analyses, DNA extracted from the recipient and donor bacteria were used as negative and positive controls, respectively. Transformants with reduced susceptibility to ampicillin, but that did not yield positive PCR products for the presence of integrons, were also tested for β-lactamase activity using a qualitative chromogenic method, with nitrocefin disks (AB Biodisk, Sweden) according to the manufacturer’s instructions. The randomly selected transformants showed reduced susceptibility to ampicillin; donors and recipient bacteria were used as positive and negative controls for β-lactamase production.

DNA sequencing

The genetic composition of the integrons of two of the donor bacteria, *A. baumannii* 064 and *S. enterica* serovar Rissen 486, as well as the flanking genomic regions of donor and transformant bacteria were determined by direct sequencing of genomic DNA and primer walking using the BigDye 3.1 cycle sequencing terminator reactions (Applied Biosystems) and an ABI3130XL genetic analyzer, as previously described [53,57]. The composition of the integrons was determined by sequencing of the integron PCR-product with primers 5′-CS and 3′-CS and an additional pair of primers for *S. enterica* serovar Rissen 486, V81 (5′-CTGGCTGCAGTATGTTCTCA-3′) and V82 (5′-GGGGCTGAGGTCTAATAG-3′). The first primers used in integron flanking regions, CS3 (5′-TCTCTACGAGTATGTTCTCAG-3′) and CS2 (5′-GGAATGGACAGCGAGGAG-3′) were designed based on the conserved regions sequence of class 1 integrons (accession number MT3819) and the flanking region sequences were obtained by primer walking, using the software Primer3 [http://fokker.wi.mit.edu/primer3/input.htm] and OligoAnalyzer [http://www.idtdna.com/Analyzer/Applications/OligoAnalyzer/]. Sequences were edited and aligned in the Sequencer v.4.2.2 program (GeneCodes, USA) and identified using the BLASTN program [http://www.ncbi.nlm.nih.gov].

The genomes of transformants (St)3 and (AbIl)3 (Table 1) were sequenced 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; and 3,667,429 bp and average sequencing depths of 15-fold, and 28-fold for (AbIl3), and (St)3, respectively. Sequence trimming, assembly, gene finding and annotation were performed with the automated GloVR-Microbe pipeline [58,59], which is part of the Cloud Virtual Resource (GloVR) appliance [60] developed in the GloVR project [http://clovr.org]. Briefly, raw sequence data were filtered and trimmed for quality and adaptor removal, and assembled with Celera Assembler [61]. 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 [62]. 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 artifacts. Fasta and annotated Genbank files of all three assemblies are available from the authors.

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

Genomic DNA was prepared in agarose blocks and digested for 3 h at 37°C with the endonuclease I-CeuI (New England Biolabs, Beverly, MA) that specifically recognize the rRNA operons [63]. The I-CeuI fragments were separated in a 1% agarose by PFGE using a CHEF-DR III apparatus (Bio-Rad, Hercules, Calif.) at 15°C, 6 V/cm with pulse time ramped from 20 s to 120 s over 11 h, followed by ramping from 60 s to 100 s for 11 h (adapted from Liu et al. [63]). 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. [64]. The 16S rRNA [65] and intII [55] probes were amplified by PCR and labeled by using a PCR digoxigenin (DIG) probe synthesis kit (Roche Diagnostics, Basel, Switzerland). A DIG luminescent detection kit (Roche) was used according to the manufacturer’s instructions. Co-hybridization for the 16S RNA and for the class 1 integrase intII was performed at 68°C.

Reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated using NucleoSpin® Triprep (Macherey-Nagel, Germany) according to the manufacturer’s instructions. Residual genomic DNA was removed by treatment with rDNase (Macherey-Nagel, Germany), followed by RNA ethanol precipitation. Reverse transcription was performed with 1 μg of RNA using the MonsterScript 1-Strand cDNA synthesis kit with random 9-mer primers (Epigenet, USA), and the resulting cDNA was used in PCR reactions. The primers HS464 and HS463a [55] amplified a 473-bp fragment of intII, and primers 16SF and 16SR [66] an approx. 1500-bp fragment of 16S rRNA. Isolated RNA was included in the PCR analyses to verify absence of DNA, and PCR targeting the 16S rRNA genes was used to confirm successful cDNA synthesis.

Biological fitness measurements

The relative fitness (W) of integron-carrying transformants was estimated by pairwise competition experiments between transformants and the untransformed recipient strain in S2-minimal medium for 24 h, as previously described [53]. Individual transformants containing one out of five distinct integrons with the following resistance genes were evaluated: bbla(M), (transformant SD3); bblaDNA-30+aadA1 (transformants [St]3, [SD1]1, SD4); dfrA12 (transformants [St]3, [SD1]1, SD4); aacA4+bblaDNA-30+aadA2 (transformant SD6); aadB (transformants SD2, [SD2]1). Selection was performed with CTX 20 μg/ml (transformant SD3), SC 10 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformant SD3), SC 10 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformants SD2, [SD2]1). Selection was performed with CTX 20 μg/ml (transformant SD3), SC 10 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformants [St]3, [SD1]1, SD4), SC 20 μg/ml (transformants SD2, [SD2]1). Nineteen to 24 competition replicates were done for each transformant. The relative fitness (W) was calculated as the ratio of the Malthusian parameter of each competitor.

Results

Location and composition of integrons in the donor bacteria

The recipient strain *A. baylyi* BD413 does not carry identifiable integrons [44] as also confirmed by our own results from PCR and genome and direct DNA sequencing of strain BD413. The availability of multichung resistant and integron-containing isolates in our own strain collection determined the initial selection of strains used as a source of donor DNA in our investigation. In order to be able to determine the stability of the integrons from the donor genomes during transformation of *A. baylyi*, the composition and genomic context of all integrons in the donor genomes was assessed by integron-specific PCR, and DNA sequencing by primer walking. PCR with primer pairs specific for the amplifi-
cation of integron gene cassettes yielded a single product, which in all cases, upon sequencing, contained at least one known antibiotic resistance gene. The direct sequencing of each PCR product indicated the presence of a single type of integron in each donor genome. In A. baumannii 064, the integron harbored a central region of 763 bp, including the aadB gene (CDS 597 bp), which is known to encode an aminoglycoside adenylyltransferase, responsible for gentamicin, kanamycin and tobramycin resistance. The integron of S. enterica serovar Rissen 486 contained a central region of 1913 bp, with two gene cassettes: dfrA12 (CDS 498 bp), and adaA2, (CDS 792 bp). The dfrA12 gene encodes a dihydrofolate reductase that confers resistance to trimethoprim, and the adaA2 gene encodes an aminoglycoside adenylyltransferase, responsible for streptomycin and spectinomycin resistance.

The genomic location of the integrons in the donor genomes was determined by PFGE. Co-hybridization of the intI1 and 16S rRNA probes was interpreted as indicating chromosomal location, whereas hybridization only with the intI1 probe was interpreted as indicating a plasmid location of the integron. A plasmid location of the integron was shown in the donors S. enterica serovar Typhimurium 490, E. cloacae C2R371 and C. freundii C16R385, while integrons in the A. baumannii 064, S. enterica serovar Rissen 486, E. coli C10R379, A. baumannii 65FFC [28] and P. aeruginosa SM [67] were located on the chromosome. The A. baumannii 064 and S. enterica serovar Rissen 486 strains both contain a plasmid, as observed by agarose gel electrophoresis (data not shown). E. fergusonii AS041A2 also harbored a plasmid and repeated PFGE results were not conclusive for the determination of the location of the integron in this strain.

Sequencing of the flanking regions of the integrons revealed that their insertion sites varied in the donors, and that the integrons were often linked to transposable elements. In S. enterica serovar Typhimurium 490, the integron was inserted in an intact Tn21-like transposon [36]. The integron of A. baumannii 064 was inserted in a Tn1721-like transposon [68,69], flanked by the insertion sequence IS26 on both sides, forming an IS26-composite transposon. The A. baumannii 65FFC integron was embedded in a defective Tn402-like transposon, flanked by a 439 bp Miniature Inverted-repeat Transposable Element (MITE) on both sides [70].

In S. enterica serovar Rissen 486, the tqaA, tqaR and tqaM genes were detected next to the 5′-CS region of the integron. The chrA gene, which codes for a putative truncated chromate ion transporter, was found flanking the 3′-CS region of the closest homologue to the tqa genes was found in the transposon Tn1721, suggesting the integron in serovar Rissen 486 may be localized inside a transposon. However, for technical reasons, we were unable to obtain additional DNA sequence by primer walking.

In P. aeruginosa SM, tqaR and tqaM genes were identified adjacent to the 5′-CS flanking region of the integron. The genes flanking the 3′-CS region could not be determined. In this case, the tqa genes were 100% identical to the genes found in the Tn5051-like transposon; also in this case indicating that the integron is located in a transposon.

The flanking regions of the integrons of C. freundii C16R385, E. cloacae C2R371, E. coli C10R379 and E. fergusonii AS041A2 were not determined.

**Interspecies transfer of integrons/gene cassettes into wild-type A. baylyi**

**Phenotypic characterization of transformants.** Natural transformation of A. baylyi BD413 by integron-containing DNA from the related species A. baumannii occurred at frequencies up to 1.6×10^{-7} transformants per recipient over a 24 h period. Natural transformation was also seen after exposure to integron-containing DNA of strains from unrelated bacterial species C. freundii, E. coli, E. cloacae, K. pneumoniae, P. aeruginosa, S. enterica serovar Rissen, and S. enterica serovar Typhimurium, at frequencies ranging from 4.4×10^{-5} to 2.1×10^{-7} (Table 2). The only two integrons containing DNA sources tested and not capable of transforming wildtype A. baylyi BD413 above the detection limit of <1 transformant per 10^{7} bacteria were two *Escherichia* isolates (E. coli C10R379 and E. fergusonii AS041A2). In addition to purified DNA, the supernatant of a heat-killed bacterial cell suspension also gave rise to bacterial transformants (Table 2) at comparable frequencies. Thus, DNA purity seems to be of limited importance. As expected, natural transformation of the competence deficient mutant A. baylyi KOC4 as a recipient did not yield transformants.

*Antimicrobial susceptibility profiles were determined for selected transformants (Table S1). The susceptibility profiles of some of the transformants were altered in ways that could not be explained by the resistance phenotypes encoded by the transferred complete integron. The hypothetical transfer of single gene cassettes rather than the entire integron could have explained the reduced susceptibility of transformants if such cassettes would have been inserted next to a host region providing promoter functions. However, none of the resistance genes, present as part of the integrons of the donor bacteria, could be identified by gene-cassette specific PCR analyses in the tested atypical transformants.*

Transfer of the insertion sequence IS{abm}a1 could hypothetically explain reduced ampicillin susceptibility in some transformants, due to induction of expression of the *A. baylyi* ampC gene [56]. However, this insertion sequence was not detected by PCR in the transformants tested. The transfer of other β-lactamase determinants unrelated to the integron could also explain reduced ampicillin susceptibility in some transformants. Observed transfer of β-lactamase was detected in two transformants resulting from exposure to DNA of A. baumannii 064 DNA (AbII4 and AbII1L); however, the molecular basis for the resistance was not determined. See footnote of table S1 for an explanation of the abbreviations of transformants.

**Genotypic characterization of transformants.** Among all the phenotypes with increased resistance observed after exposure of the BD413 strain to DNA extracted from various integron-carrying species, four transformants were shown to have acquired the complete integron from S. enterica serovar Typhimurium 490 [named SD1, (St)1, (St)2 and (St)3], and another four transformants the entire integron from A. baumannii 064 [named SD2, (AbII1), (AbII2) and (AbII3)], as confirmed by PCR (Figure S1A) and DNA sequencing. The DNA sequence of the integron and integron flanking regions of the same transformants were determined. In all cases, acquisition of complete integrons plus additional flanking DNA was shown. 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)*. Figure 1 summarizes the genetic composition of the DNA acquired by the individual *A. baylyi* transformants.

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 a copy of transposon Tn21-like, which also contained the integron. In both cases, target site duplications of 4 and 5 bp, respectively, were identified providing evidence for that the (St)1 and SD1 transformants resulted from active transposition of Tn21-like (figure S2A). The transformant SD1 also showed a partial duplication of the *merR* gene of the transposon. In transformant (St)1, the integron-containing transposon had inserted into the *A. baylyi* gene ACIAD1773, which encodes a putative transport protein permease (Figure 1A). In
Table 2. Natural transformation of *A. baylyi* with genomic DNA from different sources.

| Recipient strain | Donor strain | Mean no. of transformants (CFU) ± SD | Mean no. of recipients (CFU) ± SD | Transforms per recipients ± SD |
|------------------|--------------|----------------------------------|---------------------------------|-------------------------------|
| *A. baylyi* BD413 | Purified DNA: |  |  |  |
| *A. baumannii* 064 | (3.2 ± 3.1) × 10^{3} | > (1.5 ± 0.4) × 10^{6} | < 2.1 × 10^{-4} |
| *A. baumannii* 65FFC | (2.6 ± 2.5) × 10^{2} | > (1.6 ± 0.4) × 10^{6} | < 1.6 × 10^{-7} |
| *C. freundii* C16R385 | (5.1 ± 4.0) × 10^{5} | (4.3 ± 0.8) × 10^{8} | 1.2 × 10^{8} |
| *E. cloacae* C2R371 | (5.1 ± 2.3) × 10^{7} | (2.8 ± 0.0) × 10^{8} | 1.9 × 10^{8} |
| *E. coli* C10R379 | 0 | (7.9 ± 4.4) × 10^{9} | 0 |
| *E. coli* K71-77 | (1.0 ± 0.6) × 10^{7} | (4.7 ± 0.9) × 10^{9} | 2.1 × 10^{-7} |
| *E. fergusonii* A5041A2 | 0 | 1.7 × 10^{9} | 0 |
| *K. pneumoniae* K66-45 | (2.2 ± 3.4) × 10^{8} | (3.8 ± 1.0) × 10^{9} | 4.4 × 10^{8} |
| *P. aeruginosa* K34-73 | (3.3 ± 4.7) × 10^{1} | (3.8 ± 0.7) × 10^{8} | 7.0 × 10^{8} |
| *P. aeruginosa* SM | (4.4 ± 4.3) × 10^{1} | (1.6 ± 0.5) × 10^{7} | 2.8 × 10^{8} |
| *S. enterica* Rissen 486 | (3.1 ± 3.0) × 10^{9} | (1.7 ± 0.2) × 10^{9} | 1.8 × 10^{-7} |
| *S. enterica* Thyp. 490 | (1.4 ± 1.3) × 10^{9} | (1.4 ± 0.6) × 10^{9} | < 1.0 × 10^{-7} |
| Supernatant/lysate with DNA: |  |  |  |
| *A. baumannii* 064 | (1.7 ± 0.1) × 10^{7} | (1.9 ± 0.4) × 10^{6} | < 8.9 × 10^{-8} |
| *A. baumannii* 65FFC | (2.0 ± 1.5) × 10^{2} | (1.7 ± 0) × 10^{9} | < 1.2 × 10^{-7} |
| *P. aeruginosa* SM | (1.7 ± 0.7) × 10^{7} | (1.2 ± 0.7) × 10^{9} | < 1.4 × 10^{-7} |
| *S. enterica* Rissen 486 | (3.6 ± 0.2) × 10^{6} | (1.7 ± 0) × 10^{9} | < 2.1 × 10^{-7} |
| *S. enterica* Thyp. 490 | (3.6 ± 0.9) × 10^{1} | (8.1 ± 5.2) × 10^{8} | 4.4 × 10^{8} |
| *E. coli* C10R379 | (1.2 ± 0.7) × 10^{6} | (9.9 ± 1.4) × 10^{8} | 1.2 × 10^{7} |
| *E. fergusonii* A5041A2 | (3.1 ± 0.2) × 10^{6} | (1.3 ± 0.6) × 10^{8} | < 2.4 × 10^{-5} |
| *P. aeruginosa* SM | (4.9 ± 1.0) × 10^{1} | (5.2 ± 0.9) × 10^{8} | 9.4 × 10^{8} |
| *S. enterica* Rissen 486 | (9.4 ± 3.9) × 10^{1} | (4.7 ± 0.2) × 10^{8} | 2.0 × 10^{7} |
| *S. enterica* Thyp. 490 | (2.0 ± 0.4) × 10^{6} | (5.3 ± 1.2) × 10^{8} | 3.7 × 10^{7} |
| *A. baylyi* BD413 | 3 × 10^{5} | (6.6 ± 0.5) × 10^{8} | > 4.5 × 10^{-4} |
| *A. baylyi* SD1 | 3 × 10^{5} | (6.6 ± 0.5) × 10^{8} | > 4.5 × 10^{-4} |
| *A. baylyi* SD2 | (3.0 ± 0.1) × 10^{7} | (5.8 ± 0.3) × 10^{8} | 5.2 × 10^{8} |
| *A. baylyi* SD3 | (5.3 ± 2.8) × 10^{4} | 1.7 × 10^{6} | < 3.3 × 10^{-5} |
| *A. baylyi* SD4 | (4.1 ± 1.4) × 10^{4} | 1.7 × 10^{6} | < 2.5 × 10^{-6} |
| *A. baylyi* SD5 | (1.9 ± 0.4) × 10^{4} | 1.7 × 10^{6} | < 1.2 × 10^{-5} |
| *A. baylyi* SD6 | (1.6 ± 1.3) × 10^{5} | 1.7 × 10^{6} | < 9.6 × 10^{-5} |
| *A. baylyi* KOI | *A. baumannii* 65FFC | (3.2 ± 4.8) × 10^{5} | (1.3 ± 0.6) × 10^{8} | 2.5 × 10^{-4} |
| *P. aeruginosa* SM | (3.5 ± 0.2) × 10^{5} | 1.7 × 10^{8} | < 2.1 × 10^{-5} |
| *S. enterica* Rissen 486 | (2.7 ± 0.3) × 10^{5} | 1.7 × 10^{8} | < 1.7 × 10^{-7} |
| *S. enterica* Thyp. 490 | (7.3 ± 4.4) × 10^{4} | 1.7 × 10^{8} | < 4.4 × 10^{-5} |
| *A. baylyi* KOI | *A. baylyi* KOI (Ps1) | (2.9 ± 0.3) × 10^{5} | 1.7 × 10^{8} | < 1.8 × 10^{-3} |
| *A. baylyi* RAM | *A. baumannii* 65FFC | (5.4 ± 2.0) × 10^{4} | 1.7 × 10^{8} | < 3.3 × 10^{-5} |
| *P. aeruginosa* SM | (7.5 ± 7.6) × 10^{4} | 1.7 × 10^{8} | < 4.6 × 10^{-10} |
| *S. enterica* Rissen 486 | (2.1 ± 3.3) × 10^{4} | 1.7 × 10^{8} | < 1.3 × 10^{-9} |
| *S. enterica* Thyp. 490 | (1.9 ± 0.8) × 10^{5} | 1.7 × 10^{8} | < 1.1 × 10^{-9} |
| *A. baylyi* SD9 | *S. enterica* Thyp. 490 | (2.0 ± 1.5) × 10^{5} | (1.7 ± 0.1) × 10^{8} | 1.1 × 10^{-8} |

* Natural transformation assays were performed with purified DNA as donor source, except in the experiments with the various donor species and *A. baylyi* BD413 recipient bacteria, where supernatants of heat-treated cell suspensions were used as the sole source of DNA.

* The transformation frequency obtained with DNA from positive control source (*A. baylyi* KTG with the *ntpl* gene) was 4.7 × 10^{-4}. Negative control experiments, consisting of the same experimental conditions, with the exception of that no DNA was added, were included in each transformation assay.

* The transformation frequency is, when calculated as the frequency per exposed unit of DNA: 13 × 10^{-4} transformants per genome equivalent and 7.5 × 10^{-8} transformants per genome equivalent for *A. baumannii* 064 and *S. enterica* serovar Typhimurium 490, respectively. The genome equivalent size is based on the average size calculated from published genomes of the 2 species, as available in GenBank (Apr. 2012): 3.95 Mb and 4.99 Mb, respectively.

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Figure 1. DNA fragments acquired from *S. enterica* and *A. baumannii* by the *A. baylyi* transformants. A) Transformant (St)1 obtained after exposure to DNA of *S. enterica* serovar Typhimurium 490. B) Transformants SD1, (SD1)1, (SD1)2 and (SD1)3 obtained after exposure to DNA of *S. enterica* serovar Typhimurium 490. C) Transformants SD2, (SD2)1, (SD2)2 and (SD2)3 obtained after exposure of DNA of *A. baumannii* 064. D) Transformants (AbII)3 obtained after exposure to DNA of *A. baumannii* 064, E) transformant (St)3 obtained after exposure to DNA of *S. enterica* serovar Typhimurium 490; Gene 1 – gene coding for a bacterial regulatory, tetR family protein; Gene 2 – gene coding for a flavodoxin-like fold family protein. Class 1 integron includes the *intI1*, gene cassettes, *qac.iE*, *sulI1* and *orf5* genes.

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transformant SD1, the integron-containing transposon had inserted into gene ACIAD2320, encoding a putative proton/sodium-glutamate symport protein (Figure 1B).

Whole-genome sequence analysis of A. baylyi transformant (St)3 revealed that the integron had transferred as part of a Tn21-like transposon. The incorporated DNA was found in the transformant genome in a region containing a 54 kb 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 ACIADrRNA34 gene. The transposon present in transformant (St)3 also showed rearrangement 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, flanking the mer operon, whereas in the previously described Tn21 sequences, they are located in the 5'-CS region (Figure 1E). The rearrangement was confirmed by sequencing as well as by PCR.

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, and the insertion had also occurred in the 54 kb region indicated above.

The A. baylyi transformant SD2, obtained after exposure to A. baumannii 064 DNA, also acquired considerable stretches of genomic donor DNA-flanking the complete integron (Figure 1C). Extensive primer walking identified a crossover in the tnp gene of both the donor and recipient genomes, 5,428 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; the acquired DNA in this region included genes similar to the Tn21 transposon. The high nucleotide similarities between the tnp gene of the donor and the recipient and the characteristics of the crossover junctions suggest that recombination between homologous (also called homeologous, heterologous, or heterogenic) recombination had facilitated the acquisition of the integron-containing DNA, a process recently investigated in detail for A. baumannii donor and A. baylyi recipient cells [53].

Whole genome sequencing of the A. baylyi transformant (AbII)3 revealed that it had acquired approximately 18,625 bps of DNA sequence from the A. baumannii 064 genome, which contained a IS26-composite transposon (Figure 1D); the horizontally acquired fragment was submitted to GenBank under the accession number JX041189. The insertion contained the integron embedded in a transposon-like region including a lic (tetracycline resistance) gene and a tnpA transposase gene (74% similar to the tnpA gene of Tn1721), and further flanked on both sides by the IS26 elements. A target site duplication of 8 bp on both sides of the IS26 elements suggests transfer of the entire 18,625 bp segment occurred by transposition facilitated by IS26 sequences, into the A. baumannii 064 genome (encoding a putative membrane protein). Analysis of additional transformants, exposed to 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 a Tn1721-like transposon, and flanked by an IS26 element. Although the exact locations in the A. baylyi genome were not determined for the latter, the obtained DNA sequence suggests a similar composition and mode of transfer as for transformant AbII)3.

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 (data not shown) or PFGE, the same plasmids could not be detected in the transformants (Figure S3). The transformants SD1, (St)3, SD2 and (AbII)1 were shown by PFGE and probe hybridization to carry the acquired integron in the chromosome. It is noted that co-hybridization with the two probes targeting intI1 or the 16S rRNA 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 rRNA probe even though it is chromosomal. This is due to the opposite orientation of the rRNA operons at nucleotides 1660700–1666022 and 2941950–2947363 (access. number CR543861), leading to a 1.28 MB flanking region without the 16S rRNA gene. A. baylyi BD413 has seven rRNA operons, producing seven fragments after digestion with I-CeuI [71], which can be seen for strain BD413 and transformants (St)1 and (St)2 (Figure S3A), lanes 2, 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 S3) led to several repetitions of the experiments; however both transformants repeatedly displayed a variable chromosomal location of the integrase sequence (data not shown), also confirmed by full genome sequence of one of these transformants, (AbII)3. This variability may hypothetically be due to gene amplification, which is reported to occur in A. baylyi [72,73], followed by intra-genomic rearrangements. Rearrangements are often associated with repetitive sequences [74], which are known to represent 1.6% of the A. baylyi genome [44].

Effects of the RecBCD exonuclease. As several of the observed interspecies integron transfers appeared to have taken place through transposition, a transient existence of a linear double-stranded (ds) DNA intermediate in the cytoplasm was assumed. Such intermediates are thought to be substrates for the dsDNA-attacking exonuclease RecBCD [75,76], and recent observations suggest that RecBCD also removes dsDNA-intermediates occurring during transformation (K. Harm et al., unpublished data). We deleted the recBCD operon (and the RecBCD suppressor genes sbcCD) to obtain a wildtype-like viability; [49] in the strain BD413, yielding strain SD9 which was employed as recipient in transformation experiments. Three of the SD9 transformants showed acquisition of integron from the S. enterica serovar Typhimurium 490. The low transformation frequencies obtained with this strain do not suggest that lack of RecBCD (and/or SbcCD) protects transformable cells from transposon activity initiated by linear double-stranded DNA present in the cytoplasm.

Intraspecies transfer of integrons/gene cassettes in A. baylyi

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 1,000-fold more efficient than interspecies transfer of the same integrons (Table 2).

Crossover junctions in the A. baylyi genome sequence were detected in 6 out of 6 tested transformants, suggesting that the observed interspecies gene transfer occurred via homologous recombination. Transformant isolates (SD1), (SD2) and (SD1)3, obtained with the donor strain A. baylyi SD1, acquired at least 23,500 bp of the donor DNA. Transformant isolates (SD2), (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 6 transformants suggested that the composition of the donor DNA segments acquired in the initial interspecies transformation assay were maintained after subsequent intraspecies transformation (Figure 1A and C).

All of the tested transformants showed expected antimicrobial susceptibility profiles (Table S1) and had acquired the complete integron, which was confirmed by PCR (Figure S1C). The conserved composition of the acquired integron and the flanking regions suggest homologous recombination facilitated DNA exchange between the two A. baylyi genomes involved.

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

To determine the impact of integrons resident in the recipient bacteria on the overall transformation efficiencies, we exposed integron-containing bacteria to chromosomal DNA with integrons of different compositions. Interspecies transformation of integron-containing A. baylyi recipient cells was 10- to 100-fold more efficient than interspecies transformation of wildtype A. baylyi cells (Table 2). The presence of integron sequences in the recipient cells (A. baylyi SD2) led to efficient replacement of gene cassettes rather than to the accumulation of additional integrons or gene cassettes in all tested transformants (76 out of 76) (Figure S2B). Replacement was shown by changes in antimicrobial susceptibility profiles (Table S1) and the obtained fragment sizes by integron-specific PCR assays (Figure S1B). Recipient strains with integron-encoded _aadB_ (SD2, KOI, RAM) lost the corresponding aminoglycoside resistance profiles upon transformation by genomic DNA containing different integrons (Table S1). Gene loss and substitution of the entire integron in the transformant strain was confirmed by PCR (Figure S1B). Rearrangements of gene cassettes within the integrons were not observed among the analyzed transformants. The expression of the acquired integrase gene in A. baylyi transformant SD2 was not detected in RT-PCR analyses (Figure S4), which might explain the absence of observable recombination of gene cassettes among the limited numbers of transformants examined.

Transformation frequencies of the _recA_ mutant RAM were between 10^4-10^5-fold lower when compared to the _recA_-proficient recipient strain SD2 (Table 2), suggesting homologous recombination as mechanism responsible for efficient substitutive recombination.

**Absence of effects of the integron-encoded integrase.** To investigate whether the integrase was required for integron-acquisition or affected transformation efficiencies, experiments were repeated using the _intI1-cat-intI1_ -inactivated integron-carrying _A. baylyi_ strain KOI as a recipient. The resulting transformation frequencies were similar to those obtained with the recipient (SD2) harboring a functional integrase gene (Table 2). Of the 73 analyzed transformants, 70 acquired the entire gene cassette composition from the integron of the donor bacteria (Figure S1D). Further nucleotide analysis of the _intI1_ region of these transformants revealed that the inactivated _intI1_ region had been replaced with a functional copy from the donor bacteria in 4 out of 10 transformants after exposure to DNA from _A. baumannii_ 65FPC, 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. The lack of an effect of the _intI1-encoded integrase_ in the recombination process was also seen when the donor DNA contained an inactive integrase (Table 2); thus excluding a role of the integrase inter- and intraspecies acquisition of integrons or gene cassettes in _A. baylyi_ recipients already containing an integron.

**Relative fitness assays**

The biological cost of the acquired, integron-mediated, antibiotic resistance was determined for transformants SD3 (_blaIMP-5_, _St)_3, (SD1)_1 and SD4 (_blaOXA-21_+_aadA7, SD5 (_dfrA12+aadA2_, SD6 (_aacA4+blaOXA-21_+_aadA2_, and SD2 and (SD2)_1 (aadB). The mean relative fitness (w) of these integron-carrying strains ranged from 0.96 to 1.01 (n = 19 to 24 replicates); only transformant SD3 showed a clear negative fitness effect, with w = 0.91 (n = 23; p = 0.001). In general, no consistent major differences in fitness were observed, suggesting that the horizontal acquisitions of integrons (including the co-transformed and often extensive additional DNA regions) do not lead to immediate and severe growth inhibition of transformant cells.

**Discussion**

Horizontal gene transfer (HGT) is a key driver of bacterial adaptation and evolution [77–79]. A number of studies demonstrate that a significant amount of bacterial genomes are affected by HGT events [80–83]. In this study we examined to what extent DNA substrates and natural transformation can lead to interspecies transfer of distinct genetic elements encoding site-specific recombination mechanisms.

Annealing of complementary DNA strands in the cytoplasm of recipient bacteria is considered necessary for recircularization and hence stable uptake of plasmids in competent bacterial species [84] (K. Harms, unpublished data). Drawing on this general observation, we hypothesized that annealing of other single-stranded DNA 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 double-strand DNA fragments could lead to integration of the elements into the host chromosome. To what extent natural transformation of bacteria can facilitate integragenomic mobility of genetic elements being part of species foreign, linear DNA fragments can be tested.

Indeed, we provide experimental data demonstrating that natural transformation can facilitate interspecies transfer of integrons and transposons that is not limited by the genetic relatedness of the donor with the host. Exposure of _A. baylyi_ to DNA of an integron-containing _Salmonella_ strain led to horizontal transfer of its integron due to transposition of a Tn21-like transposon that contained the integron. The Tn21-like transposon was found as single inserts at different chromosomal locations in 3 different examined transformant genomes. Exposure of _A. baylyi_ to DNA of an integron containing _A. baumannii_ strain also led to transformants with single chromosomal integrations of the integron. The integrations occurred by either DNA sequence similarity-based homologous recombination, or by genetic linkage to integration of the elements into the host chromosome. To what extent natural transformation of bacteria can facilitate integragenomic mobility of genetic elements being part of species foreign, linear DNA fragments can be tested.

**Interspecies transfer into integron-free _A. baylyi_ cells**

Purified DNA substrates of _A. baumannii_ 064 and _S. enterica_ serovar Typhimurium 490 transformed _A. baylyi_ cells at frequencies of 10^−5 and 10^−4 over a 24 h time period. In all examined cases, the composition and order of gene cassettes in the donor bacteria was maintained in the transformant cells. Also DNA lyases could transform _A. baylyi_ cells suggesting variability in DNA purity is of minor importance for HGT, as observed previously [51].
DNA sequencing of the flanking regions of the integrons in 6 initial transformants obtained after exposure to species-divergent donor DNA verified 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 also 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 bps long. The sequencing of the acquired DNA in 6 transformants obtained after exposure to DNA of various species, as well as Southern hybridization of a total of 8 transformants, revealed that integration had occurred in different regions of the A. baylyi genome.

Most interestingly, stable integration of the integron containing DNA in the A. baylyi chromosome had occurred by several mechanisms, depending of 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; such as transposon Tn21-like. Integrons are often embedded in transposon Tn21 [36], and many different gene cassette arrays have been reported for integrons present in Tn21-like transposons [85]. The horizontal dissemination of this transposon is a major contributor to the dissemination of integrons. Here we have experimentally demonstrated that natural transformation can facilitate interspecies movement of this transposon. For a related species (A. baumannii DNA donor), homologous recombination-based insertions were observed, as expected [53]. However, in this latter exposure scenario, also site-specific recombination due to IS26-associated transposition was observed. Similarly to Tn21-like, IS26 elements are often associated with antibiotic resistance genes and presumed active in Acinetobacter [86]. There are several reports of IS26-associated integrons [87,88], and transposition of IS26-composite transposons has been suggested [89]. The presence in the recipient 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 [88,90]. A previous bioinformatics-based study of Partridge et al. [41] also indicated transposition as a possible mechanism of integron movement. However, no experimental tests were performed.

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 utilized. For instance, both model and applied systems can be developed using small DNA fragments with site-specific recombination functions as donor DNAs. Moreover, possible interactions between recombineses encoded by the incoming DNA and host enzymes should be investigated. For example, the A. baylyi ADP1 strain harbors two 2 prophages, a number of transposases, and 6 copies of IS1236, with two of them flanking Tn5613 [44]. The exposure of A. baylyi cells to the various DNA substrates resulted in surprisingly high transformation frequencies, 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 integrons. The observation of variable changes in susceptibility patterns, after exposure to various sources of bacterial DNA, suggest the broad potential of and the presence of a yet not described range of transferable resistance determinants in bacteria, of unknown mechanistic and genetic nature. 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. In two examined cases, changes in resistance profile were due to the acquisition of β-lactamases as determined by the nitrocefin test. The broader effects of the composition of the A. baylyi genome after the heterologous recombination with DNA from the related species A. baumannii will be determined in a separate study. A recent study by Mell et al. [91] reported transfer of numerous chromosomal polymorphisms between Haemophilus influenzae genomes after natural transformation.

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

Natural transformation of the 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 acquisitions of integrons that take place at low initial frequencies can be followed by high-frequency interspecies dissemination. Interestingly, 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 integron (Figure S2B). A role of the intI1 integrase was not observed in our studies.

Transformation experiments performed with recipient cells containing an integron with the integrase gene inactivated (strain KOI), and also with recipient cells containing an integrin, but with the recA gene inactivated (strain RAM) that resulted four orders of magnitude reduction in transformation frequencies, confirm the role of homologous recombination [53,92] in the replacement of integrons and gene cassettes in these recipient bacteria. Partridge et al [41] also suggested replacement of a gene cassette array in Pseudomonas 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 can therefore likely contribute to the formation and spread of chromosomally-located complex mosaic regions, often formed by several resistance genes and mobile elements [93].

Recent publications demonstrate that many integrase genes in integrons carry LexA binding sites in the vicinity of their promoter regions and can be controlled by the host LexA protein (transcriptional repressor of the SOS response [94,95]). In organisms harboring lexA alleles, SOS induction can lead to increased integrase activity resulting in increased frequencies of cassette rearrangements [94,95]. SOS induction of the expression of the integrase by both conjugation [95] and natural transformation [96] has been recently shown in V. cholerae. In contrast, and unlike many Enterobacteriaceae, the SOS response in A. baylyi is not regulated by a LexA orthologue [97]. The integrase gene in our recipient strain is therefore derepressed or regulated by other unknown factors, as expression was not found in the RT-PCR.
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 will occur much more frequently than integrase-catalyzed cassette recombination events. Therefore, rare rearrangements of gene cassettes may have occurred in our model system but would not be detectable in the limited number of transformants that were genetically characterized. The recent work published by Baharoglu et al. [95] on conjugative induction of SOS and its ability to trigger cassette recombination showed that cassette rearrangements occurred at very low frequencies (e.g. \(10^{-7}\)) 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 [98].

General considerations

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 [99]. Our relative fitness measurements suggest that the acquired integrons and the resulting changes in resistance profiles do not cause major fitness reductions of transformants. It is noted that the fitness measurements represent the combined effect of all transferred DNA segments, as well as effects on the transformant genome due to insertion effects.

Natural competence is described in at least 60 species of bacteria, including several species present in clinical environments [100]. However, the proportion of species able to acquire DNA by natural transformation remains to be clarified, as many species or strains may have the capacity without such being detectable under laboratory conditions that are limited in sensitivity, time and by environmental variables [100]. The occurrence of naturally transformable species within the pool of uncultivable bacteria also remains to be fully explored. Interestingly, several species or strains, including E. coli, A. baumannii and Pseudomonas stutzeri, regarded as non-competent have later been shown to be naturally transformable under certain circumstances [101–104]. Natural transformation could, for instance, be responsible for the dissemination of chromosomal integrons in P. aeruginosa [105]. Recently, the acquisition of (synthetic) gene cassettes by natural transformation in a Pseudomonas species [39] was also shown; indicating that homology-independent DNA acquisitions might be more general phenomenon. It is also worth noting that chromosomal super-integrons are mainly found in Vibrio species, which are naturally transformable [9,100].

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 integron-containing transposons) through natural transformation provided the transferred DNA fragments encodes functions for site-specific 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 transposons, and insertion sequences through natural transformation. Hence, presenting a new pathway for horizontal dissemination of antimicrobial resistance determinants present in integrons embedded in transposons.

Gene reference numbers

The reference numbers for genes mentioned in the text include: AC1AD0480 (2881081), AC1ADI773 (2879997), AC1AD3230 (2879415), AC1ADRnaset_34 (2877688), Tn4286 (5741162), amprC (2800712), dfrA1 (5741160), intII (11934322) recA (2879746), recB (2879747), recC (2879748), recD (2879749), tnpA (2879604), sbcC (2879936), sbcD (2879937), 16S rDNA (2880271) GeneID and blaMDR (AP289012), blaOXA-34+aadA1 (AY534545), aacA7-blaSEN +aadA1 (DQ219465), blaPM17+tetr+aarA+aarB-blase1 (EN397623), IsbBal (AY738396), MTEx [FJ010083], Tn21-like (AM991977) and Tn9031-like (AJ867812) accession number from the NCBI Genbank database. The IS26-66 composite transposon was submitted to GenBank with the accession number JX041889.

Supporting Information

Figure S1 Agarose gel electrophoresis of class 1 integron PCR-products. A) Lane 1 – A. baumannii 064 (donor strain); 2–5 – transformants from A. baumannii 064, 4,10.1, 4,31.1, 4,34,1 and 4,46,1 respectively; 6 – S. enterica serovar Typhimurium 490 (donor strain); 7–10 – transformants from S. enterica serovar Typhimurium 490, 2,1.1, 2,3,6, 2,2,4,5 and 2,2,6, respectively; 11 – A. baylyi BD413 (recipient strain); 12–1 Kb Plus DNA ladder (Invitrogen). B) Lane 1 – 1 Kb Plus DNA ladder (Invitrogen); 2 – A. baumannii 65FFC (donor strain); 3–5 – transformants from A. baumannii 65FFC, SD3, [SD2]Ab1 and [SD2]Ab2, respectively; 6 – C. freundii C16R385; 7–9 transformants from C. freundii C16R385, [SD2]Cf1, [SD2]Cf2, [SD2]Cf3, respectively; 10 – E. cloacae CR2371; 11–13 transformants from E. cloacae CR2371, [SD2]Ecl1, [SD2]Ecl2, [SD2]Ecl3, respectively; 14 – E. coli C10R379; 15–17 – transformants from E. coli C10R379, [SD2]Ecl1, [SD2]Ecl2, [SD2]Ecl3, 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]Ef1, [SD2]Ef2, [SD2]Ef3, respectively; 25 – P. aeruginosa SM (donor strain); 26–28 – transformants from P. aeruginosa SM, SD6, [SD2]Ps1, [SD2]Ps2, respectively; 29 – S. enterica serovar Rissen 486 (donor strain); 30–32 – transformants from S. enterica serovar Rissen 486, SD6, [SD2]Sr1, [SD2]Sr2, respectively; 33 – S. enterica serovar Typhimurium 490 (donor strain); 34–36 – transformants from S. enterica serovar Typhimurium 490, SD4, [SD2]Sr1, [SD2]Sr2, respectively; 37 – transformant SD2 (recipient bacterium); 38– A. baumannii 65FFC (negative control). C) Lane 1 – transformant SD1 (donor strain); 2–4 – transformants from SD1, [SD1]D1, [SD1]D2, [SD1]D3, respectively; 5 – transformant SD2 (donor strain); 6–8 – transformants from SD2, [SD2]D1, [SD2]D2, [SD2]D3, respectively; 9 – transformant SD3; 10–12 – transformants from SD3, [SD3]D1, [SD3]D2, [SD3]D3, respectively; 13 – transformant SD4; 14–16 transformants from SD4, [SD4]D1, [SD4]D2, [SD4]D3, respectively; 17– transformant SD5; 18–20 – transformants from SD5, [SD5]D1, [SD5]D2, [SD5]D3, respectively; 21 – transformant SD6; 22–24 transforms from SD6, [SD6]D1, [SD6]D2, [SD6]D3, respectively; 25 – A. baulyi BD413 (recipient strain); 26 – 1 Kb Plus DNA ladder (Invitrogen). D) Lane 1 – 1 Kb Plus DNA ladder (Invitrogen); 2 – A. baumannii 65FFC (donor strain); 3–5 – transformants from A. baumannii 65FFC, [KOI]Ab1, [KOI]Ab2, [KOI]Ab3, respectively; 6 – P. aeruginosa SM (donor strain); 7–9 – transformants
from *P. aeruginosa* SM, [KOI][P1], [KOI][P2], [KOI][P3], respectively; 10 – *S. enterica* serovar Rissen 486 (donor strain); 11–13 – transformants from *S. enterica* serovar Rissen 486, [KOI][St1], [KOI][St2], [KOI][St3], respectively; 14 – *S. enterica* serovar Typhimurium 490 (donor strain); 15–17 – transformants from *S. enterica* serovar Typhimurium 490, [KOI][St1], [KOI][St2], [KOI][St3], respectively; 18 – transformant KOI (recipient bacterium); 19 – *A. baylyi* BD413 (negative control). (TIF)

Figure S2 Schematic presentation of the horizontal acquisitions. A) Acquisition of a transposon by transposition with duplication of sequences around insertion site; B) Acquisition or substitution of gene cassettes by homologous recombination occurring between conserved regions of a class 1 integron. (TIF)

Figure S3 PFGE and Southern blot hybridization. A) Pulse-field gel electrophoresis (PFGE) of I-Cell-digested total DNA of recipient, donor and transformant bacteria; B) corresponding Southern hybridization with 16S rRNA probe; C) corresponding Southern hybridization with intI1 probe. Lane 1 – lambda PFGE 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, (St1), SD1, (St2), (St3), respectively; 8 – *A. baumannii* 064 (donor); 9–12 – transformants from exposure to DNA of *A. baumannii* 064, SD2, (AbI1), (AbI2) and (AbI3), 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 PFGE marker (New England Biolabs). (TIF)

Figure S4 RT-PCR of the class 1 integrase RNA in transformant *A. baylyi* SD2. Lane 1 (left) – SmartLadder (Eurogentec); 2–3 – PCR targeting the intI1 gene, 2 – cDNA; 3 – RNA; 4–5 – PCR targeting the 16S rRNA gene, 4 – cDNA; 5 – RNA. For primers description, see Material and Methods. (TIF)

Table S1 Antimicrobial susceptibility of *A. baylyi* transforms determined by the E-test method. (DOC)

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Author Contributions

Conceived and designed the experiments: SD KH PJ GJdS KMN.Performed the experiments: SD KH WFF. Analyzed the data: SD WFF. Wrote the paper: SD GJdS KMN.

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