The native *Pseudomonas stutzeri* strain Q chromosomal integron can capture and express cassette-associated genes

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The integron-gene cassette system contributes to multiple antibiotic resistance in bacteria and is likely to be of broader evolutionary significance. However, the majority of integron diversity consists of chromosomal integrons (CIs), with mostly unknown phenotypes, which are poorly characterized. A pUC-based reporter plasmid (pUS23) was developed containing a recombination site [aadB 59 base element (59-be)] upstream of promoterless aadB [gentamicin (Gm) resistance] and *gfp* (green fluorescence) genes, and this construct was used to investigate the recombination and expression activities of the CI in *Pseudomonas stutzeri* strain Q. Electroporation of pUS23 into *P. stutzeri* Q gave ampicillin-resistant transformants, which yielded Gm\(^R\) green fluorescent recombinants after plating on Gm medium. Site-specific integration of pUS23 at *attl* was detected by PCR in 8\% of Gm\(^R\) colonies and the frequency of *attl* integration was estimated as 2.0 x 10\(^{-8}\) per *P. stutzeri* O(pUS23) cell. RT-PCR confirmed integron-mediated expression of *aadB* in one recombinant strain (Q23-17) and a promoter (P\(_c\)) was localized to the 5' end of the *intI* gene. The integrated pUS23 and flanking integron DNA were cloned from genomic DNA of strain Q23-17 and sequenced, confirming that site-specific integration of the entire reporter plasmid had occurred at the *attl* site. An insertion sequence (ISP\(_{is5}\); ISP\(_{5}\) family) was discovered in the vector backbone of the reporter plasmid integrated at *attl* and also in a pUS23 derivative recovered as a plasmid in *Escherichia coli* JM109. This is the first demonstration that wild-type CIs can capture gene cassettes and express cassette-associated genes.

### INTRODUCTION

Integrons and gene cassettes function together as a distinctive genetic system (Fig. 1). Gene cassettes typically consist of a single promoterless gene and an associated recombination site (termed 59 base element, 59-be or *attC*). The total pool of cassette-associated genes in natural bacterial populations (gene cassette metagenome) is a significant source of genetic novelty (Holmes et al., 2003a; Michael et al., 2004; Stokes et al., 2001), and since individually packaged genes create the possibility of combinatorial genetics, the significance of the gene cassette metagenome is potentially far reaching. However, gene cassettes are dependent on externally supplied functions for both mobilization and expression. These functions are supplied by integrons (Hall & Collis, 1995; Rowe-Magnus & Mazel, 2001). The key features of an integron are a recombination site (*attl*), an enzyme (integron integrase, IntI) that catalyses site-specific recombination between cassette and integron sites (*attl* x 59-be or 59-be x 59-be) and a cassette promoter (P\(_c\)). Collectively, these give an integron the capacity to acquire gene cassettes, express gene-cassette-associated ORFs (gcORFs) and rearrange the transcriptional order of gcORFs through repeated integration and excision of gene cassettes.

Interpretation of the significance of the gene cassette metagenome is dependent on understanding the distribution and diversity of integrons. Under the selective pressure of intensive antibiotic use, class 1 integrons have used the cassette metagenome to engineer the phenotype of multiple antibiotic resistance (Hall & Collis, 1998; Rowe-Magnus et al., 2001). Examination of genome sequence data and targeted environmental surveys have shown that integrons are diverse and present in ~5\% of bacterial genomes (Nemergut et al., 2004; Nield et al., 2001; Rowe-Magnus et al., 2001). Given the size of the gene cassette reservoir and the relative frequency of integrons, we might expect that the integron–gene cassette system has also engineered phenotypes other than antibiotic resistance. Surprisingly, such

### Abbreviations:

- Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Tc, tetracycline; 59-be, 59 base element; CI, chromosomal integron; gcORF, gene-cassette-associated ORF; MI, mobilized integron.
- The GenBank/EMBL/DDBJ accession numbers for the sequences of IS element IS\(_{ps5}\) and of pUS23 reported in this paper are AY894752 and AY894753, respectively.
evidence is hard to find, suggesting that other integron classes have been less successful in this regard than class 1 integrons. A contributing factor may be that not all integrons have the same opportunities to access the gene cassette metagenome.

Integrons have been observed in a diverse range of genetic elements, including transposons, plasmids, genomic islands, chromosomes and combinations thereof (Boyd et al., 2002; Hall & Collis, 1998; Hochhut et al., 2001; Liebert et al., 1999; Rowe-Magnus et al., 2001; Szczepanowski et al., 2004). The typical cassette array characteristics of those integron classes associated with mobile elements (mobilized integrons, MI) differ from those integrons that are only known from bacterial chromosomes (chromosomal integrons, CI). In comparison to MIs, CIs tend to contain larger cassette arrays, have significantly greater uniformity in 59-bes and predominantly contain cassette-associated genes of unknown function. These differences are likely to reflect different capacities to move to new sources of gene cassettes, but do they also reflect diversity in integron functions? It has been suggested that class 1 integrons have a greater capacity to express cassette-associated genes than other integron classes (Hanau-Bercot et al., 2002) and even within class 1 integrons different versions of Pc are known, which vary significantly in their promoter strength (Collis & Hall, 1995; Levesque et al., 1994). The issue of variation in integron function is particularly significant in the case of CIs since they can form long-term associations with specific bacterial lineages (Gillings et al., 2005; Rowe-Magnus et al., 2003, 2001). Are such integrons fully functional or primarily cassette reservoirs?

Available data are limited, but indicate that CIs share the site-specific recombination functions of class 1 (Collis et al.,

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**Fig. 1.** Structure of pUS23 reporter plasmid and mechanism of capture by InPstQ. (a) Reporter construct pUS23: bla, β-lactamase (ApR); pUC ori, pUC19 origin of replication; P lac, lac promoter of pUC19; T1T2, rmBT1T2 transcription terminators; aadB 59-be (attC site) associated with aadB gene; aadB, promoterless aminoglycoside adenylyltransferase (GmR KmR TbR); gfp, promoterless GFP (mutant 3). All restriction sites indicated are unique, except KpnI, and are abbreviated as follows; E, EcoRI; K, KpnI; Sm, Smal; B, BamHI; X, XbaI; S, SalI; P, PstI; BglII; A, Apal; H, HindIII. (b) P. stutzeri Q transformant containing pUS23 and InPstQ. Expression of IntIPstQ integrase leads to site-specific recombination between the aadB 59-be and the attIPstQ site, and integration of the plasmid into InPstQ. Also indicated are 59-bes associated with the native gene cassettes of P. stutzeri Q (BGC001–003) and the integron-associated promoter P c. The integrase promoter is not shown, but is expected to be located within or near the attIPstQ site. (c) Recombinant strain containing pUS23 integrated at attI. The aadB and gfp genes are expressed from the integron-associated P c promoter. Note that the wild-type PstQ integron contains 10 gene cassettes, of which only the first three are indicated in this figure.
and class 3 (Collis et al., 2002a) integrons. The integron integrases from CIs of various species have been expressed in Escherichia coli and shown to catalyse site-specific recombination between attI or 59-be sites (Drouin et al., 2002; Holmes et al., 2003b; Leon & Roy, 2003; Rowe-Magnus et al., 2001). However, changes in cassette arrays within a single strain have not been observed over time in lab cultures (unpublished data) and it is notable that integration, excision or P_c-mediated expression of gcORFs have not yet been reported in a wild-type CI. A particularly significant issue is the extent to which cassette-associated genes in the very large cassette arrays of some CIs can be expressed. We describe here an approach for addressing these issues. A model gene cassette was constructed by inserting 59-be and promoterless reporter genes into a pUC19 backbone. When transformed into cells containing integrons, this reporter cassette (pUS23) can provide evidence for both integron integrase and P_c functionality. We used the reporter pUS23 to investigate the CI in Pseudomonas stutzeri strain Q (InPstQ) (Holmes et al., 2003b) and report here the first evidence of IntI-mediated gene capture at attI and expression from P_c in a wild-type CI.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains are described in Table 1. All bacteria were grown aerobically in LB medium (Sambrook & Russell, 2001) at 37°C. Gentamicin (Gm) was added at 10 μg ml⁻¹ for E. coli strains and at 3 μg ml⁻¹ for Pseudomonas strains unless indicated otherwise. Chloramphenicol (Cm) was added at 25 μg ml⁻¹ and tetracycline (Tc) at 10 μg ml⁻¹ for E. coli strains. Ampicillin (Ap) was added at 100 μg ml⁻¹ for both genera. Minimal inhibitory concentrations (MICs) were determined in broth cultures inoculated at an OD₆₀₀ of approximately 0.05 and grown for 16 h.

**Microscopy.** Cells from late-exponential-phase broth cultures (OD₆₀₀ = 1.0–1.5) were washed in sucrose buffer (0.3 M sucrose, 1 mM MgCl₂, 1 mM HEPES, pH 7), concentrated 10-fold and 5 μl samples were mounted on pads of 1% agarose in the same buffer. An Olympus BX60 microscope with a Chroma filter set 41018 (Chroma Technologies) was used for fluorescence microscopy. Images were captured with a SenSys digital CCD camera (Photometrics) and processed using V for Windows software (Digital Optics). An exposure time of 50 ms was used for all experiments.

**General DNA techniques.** Plasmids and oligonucleotides used are described in Table 1 and Table 2, respectively. Plasmid DNA extraction, restriction digestion and ligation were done by standard methods.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. stutzeri Q (PstQ)</td>
<td>Contains integron InPstQ, consisting of intIPstQ, attIPstQ and 10 gene cassettes (BGC001–BGC030)</td>
<td>Holmes et al. (2003b)</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>rccA1 supE44 endA1 hsdR17 gyrA96 relA1 thi D (lac-proAB) F’[traD36 proAB + lacZ Δ(lac-proAB)]</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>P. stutzeri Q (pUS23)</td>
<td>Ap² transformant of P. stutzeri Q</td>
<td>This study</td>
</tr>
<tr>
<td>Q23-7</td>
<td>Ap² Gm² GFP⁺ derivative of P. stutzeri Q (pUS23); contains pUS23 integrated at attIPstQ</td>
<td>This study</td>
</tr>
<tr>
<td>Q23-17</td>
<td>Ap² Gm² GFP⁺ derivative of P. stutzeri Q (pUS23); contains pUS23 derivative integrated at attIPstQ</td>
<td>This study</td>
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<td>Q23-25</td>
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<td>Q23-10</td>
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<td>Q23-13</td>
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<td><strong>Plasmids</strong></td>
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<td>pUC19</td>
<td>2.7 kb, Ap²; E. coli cloning vector</td>
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<tr>
<td>pACYC184</td>
<td>4.2 kb, Cm² Tc²; E. coli cloning vector</td>
<td>Chang &amp; Cohen (1978)</td>
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<td>pMAQ105</td>
<td>8.8 kb, Ak² Ap² Cm² Gm² Km² Nt² Tb²; integron fragment from pBWH301 cloned in pACYC184</td>
<td>Bunny et al. (1995)</td>
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<td>pTGFP-2</td>
<td>5.0 kb, Ap²; Pst·atpE·gfpmut3</td>
<td>Hansen et al. (2001)</td>
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<td>pUS21</td>
<td>2.8 kb, Ap²; amplified aadB 59-be (105 bp) at Xbal/PstI site in pUC19</td>
<td>This study</td>
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<tr>
<td>pUS22</td>
<td>4.0 kb, Ap² Gm² GFP⁺; amplified aadB (567 bp) and gfpmut3 (747 bp) at PstI/HindIII site in pUS21</td>
<td>This study</td>
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<tr>
<td>pUS23</td>
<td>4.3 kb, Ap² Gm² GFP⁺; amplified rnrBT1TI (267 bp) at Xbal/SalI site in pUS22</td>
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<tr>
<td>pUS40</td>
<td>16.6 kb, Cm² Ap² Gm²; a 12.3 kb Std fragment of Q23-17 genomic DNA cloned at EcoRV site in tetA gene of pACYC184</td>
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Table 2. Oligonucleotides

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<th>Primer</th>
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<th>Target†</th>
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<td>NVC27</td>
<td>GGGTCTAGAGTGCAGTTGCGTGGCGTTTCAG</td>
<td>aadB 59-be in pMAQ105 (F)</td>
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<tr>
<td>NVC28</td>
<td>GGGCTGCAAGATTGCTGTAAGTGGGCTGTAAGAAGT</td>
<td>aadB 59-be in pMAQ105 (R)</td>
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<tr>
<td>NVC33</td>
<td>TGTGGATTGAGGCTAGGATGATGACCCAGGAGAAGAAC</td>
<td>aadB in pMAQ105 (F)</td>
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<td>NVC34</td>
<td>GACGGTACCGTTAACGCGAGTCGACCTGAAAAGGAGAAGAAC</td>
<td>aadB in pMAQ105 (R)</td>
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<tr>
<td>NVC35</td>
<td>AAGGGTACAAAGGAGGTTTTTATGCTAAGAAGAAGAAGAAC</td>
<td>gfp mut3 gene in pTFG2 (F)</td>
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<td>NVC36</td>
<td>AAAAGGCTGAAGGAGGTTTTTATGCTAAGAAGAAGAAGAAC</td>
<td>gfp mut3 gene in pTFG2 (R)</td>
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<tr>
<td>NVC37</td>
<td>GTGTACAGAATCAAAATTTATGCTAAGAAGAAGAAGAAC</td>
<td>rrsB T1T2 terminators in JM109 (F)</td>
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<td>NVC38</td>
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<td>rrsB T1T2 terminators in JM109 (R)</td>
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<td>NVC39</td>
<td>GTGTACAGAATCAAAATTTATGCTAAGAAGAAGAAGAAC</td>
<td>bla gene in pUS23 (F)</td>
</tr>
<tr>
<td>NVC40</td>
<td>GAAAACCTGAGTGGGATTTTATGCTAAGAAGAAGAAGAAC</td>
<td>ori region in pUS23 (R)</td>
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<td>NVC43</td>
<td>GTGTACAGAATCAAAATTTATGCTAAGAAGAAGAAGAAC</td>
<td>bla gene in pUS23 (R)</td>
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<td>NVC44</td>
<td>GTCATGACGCGACGAGAAGAAC</td>
<td>ori region in pUS23 (F)</td>
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<td>NVC45</td>
<td>GTCATGACGCGACGAGAAGAAC</td>
<td>in vitro activity of PstQ (F) relative to cassettes aadB (R)</td>
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<td>NVC71</td>
<td>CTAAGGAATCCTAGTCAAGGAGATAC</td>
<td>ORF in pStQ BGC001 cassette (F)</td>
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<td>NVC74</td>
<td>ATGAAACCATCATGCGACAGCC</td>
<td>ORF in pStQ BGC001 cassette (R)</td>
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<td>NVC75</td>
<td>TACCGAGTTAAGCAGTTAAGAAGAAC</td>
<td>ORF in pStQ BGC030 cassette (F)</td>
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<td>AJH125</td>
<td>AGTATCGTCACCACCATCCTC</td>
<td>ori region in pUS23 (R)</td>
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<td>NVC89</td>
<td>CCTTTTAGATTTTTTACAG</td>
<td>Between bla and ori in pUS23</td>
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<tr>
<td>NVC90</td>
<td>TCTCATGACGACGGAATTTTAC</td>
<td>in vitro activity of PstQ (F) relative to cassettes aadB (R)</td>
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<td>AJH21</td>
<td>ATGAAAGATGATTGTTAGGCGG</td>
<td>attI of PstQ (F) relative to cassettes aadB (R)</td>
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<tr>
<td>NVC91</td>
<td>CATCCACCTCCAAACAGGTCG</td>
<td>aadB (R)</td>
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<td>AGCGGCATCTGAGCCTCAACCTTTC</td>
<td>aadB (R)</td>
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<tr>
<td>NVC108</td>
<td>GTTCGACTGAGTCGACCTTAC</td>
<td>BGC001 cassette in PstQ (R)</td>
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<td>TTGCTGATTTGCTGATTGTC</td>
<td>ori region in pUS23 (R)</td>
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<td>NVC113</td>
<td>TTGCAAGCGAGCAGATTGAC</td>
<td>bla gene in pUS23 (R)</td>
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<tr>
<td>NVC114</td>
<td>CCTCAGCTGATTAAGGATTCG</td>
<td>bla gene in pUS23 (R)</td>
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</table>

*Restriction sites are underlined.
†(F) indicates forward and (R) indicates reverse primers.

**Methods.** (Sambrook & Russell, 2001). Qiaquick columns (Qiagen) were used to purify DNA for ligations and sequencing. Molecular biology enzymes were from Genesearch (New England Biolabs). DNA sequencing of plasmids and PCR products was done by the dye-terminator method on an ABI 3730 machine (SUPAMAC facility, University of Sydney). Genomic DNA was extracted by a CTAB (N-cetyl-N,N,N-trimethylammonium bromide) method as follows. Cells from 50 ml broth cultures were resuspended in 5 ml STE buffer (1 M NaCl, 10 mM Tris, 1 mM EDTA), then CTAB (0.1% w/v) was added and incubated at 65°C for 30 min. Cholorform/isooamil alcohol (24:1 ratio, 5 ml) was added, mixed thoroughly and the lysate incubated on ice for 30 min before centrifugation (20000 g, 10 min, 4°C). The DNA-containing supernatant was further purified with phenol/chloroform and ethanol (Sambrook & Russell, 2001). Both *E. coli* and *Pseudomonas* strains were transformed by electroporation (2–5 kV, 25 μF, 200 mΩ, 0.2 cm gap cuvettes), essentially using standard methods (Sambrook & Russell, 2001), except that *Pseudomonas* cells were harvested in late exponential phase (OD 600 ~1.0) and wased and resuspended in sucrose buffer (see above). Recovery after electroporation was in LB medium (1 ml) for 1 h (*E. coli*) or 2 h (*Pseudomonas*) at 37°C before cells were plated on antibiotic media.

**PCR.** PCR was done in 25 μl reactions containing *Pfu* polymerase (1 U), dNTPs (0.2 mM) and primers (1 μM each) in a buffer containing Tris/HC1 (20 mM, pH 8.8), KCl (10 mM), (NH4)2SO4 (6 mM), MgSO4 (2 mM), Triton X-100 (0.1%) and bovine serum albumin (0.1 mg ml−1). The template for PCR was either purified DNA (5–10 ng) or cells (~0.5 µl) on a 10 μl tip dipped into the PCR mix several times. Thermocycling consisted of initial denaturation (94°C, 5 min), then 30 amplification cycles (94°C for 30 s, 65°C for 30 s, 72°C for 1 min per) and a final extension (72°C for 5 min).

**Construction of pUS23 reporter plasmid.** The reporter plasmid pUS23 was constructed by PCR in *E. coli* JM109 using the pUC19 vector backbone. A fragment including the aadB 59-be was amplified from pMAQ105 with the primers NVC27/NVC28 and cloned into Xbal/PstI-cut pUC19 to yield pUS21. The aadB gene (NVC33/NVC34 amplicon from pMAQ105) and gfp mut3 gene (NVC35/NVC36 amplicon from pTFG2) were digested with PstI/KpnI and KpnI/HindIII, respectively, and joined to *PstI/HindIII*-digested pUS21 in a three-way ligation, yielding pUS22. The rrsB T1T2 terminators from the JM109 chromosome were amplified with NVC37/NVC38, digested with Xbal/SalI and ligated into Xbal/SalI-cut pUS22 to yield pUS23 (Fig. 1). The expected structure and function of pUS23 in *E. coli* were confirmed by restriction mapping, sequencing of the reporter region and phenotypic tests (Gm MIC for aadB; fluorescence microscopy for gfp).

**Introduction of pUS23 into P. stutzeri Q and selection of recombinants.** Plasmid pUS23 (1 µg) was electroporated into *P. stutzeri* Q (5 × 108 cells) and after recovery in 1 ml LB broth (2 h), dilutions were plated on LB-Ap. After 4 days, ApR colonies from one plate (several hundred) were pooled into 5 ml LB-Ap culture.
broth and grown to stationary phase (2 days, approx. $3 \times 10^8$ cells ml$^{-1}$). Dilutions were spread on LB-Gm plates to detect activation of $aadB$ and $gfpmut3$, and on LB plates to determine total viable count. Plain LB medium was used for determining the total count of P. stutzeri Q(pUS23) cells due to the instability of the intermediate P. stutzeri Q(pUS23) strain and its failure to reliably form single colonies on LB-Ap plates. Colonies appearing on Gm plates were subcultured to patches on the same medium and screened by PCR (below). The whole procedure from electroporation to Gm$^R$ screening was repeated three times and the mean frequencies of transformation to Ap$^R$ (per µg DNA) and conversion to Gm$^R$ (per P. stutzeri Q cell) were calculated. Under the conditions used, the spontaneous frequencies of Ap$^R$ and Gm$^R$ in controls without plasmids were $3 \times 10^{-7}$ per cell and $< 10^{-10}$ per cell, respectively (mean of three experiments).

**PCR screening for attI recombination.** Ap$^R$ Gm$^R$ colonies were initially screened for the presence of the attIPstQ-$aadB$ integron recombination junction by PCR using the primers NVC70/NVC71. This separated the transformants into two Gm$^R$ groups, those that putatively contained pUS23 integrated at attIPstQ and those that did not. A representative Gm$^R$ strain of each group from all three experiments was purified by restreaking, yielding six Ap$^R$ Gm$^R$ P. stutzeri Q(pUS23) derivatives (strains Q23-7, Q23-10, Q23-12, Q23-13, Q23-17 and Q23-25) for further analysis. A second PCR screen for the $aadB$ 59b-ΔC-gene fragment was performed on these six strains using the primers NVC27/NVC73. Where evidence of pUS23 integration was obtained, both junction sequences were determined by purification and direct sequencing of the PCR products.

**Hybridization analysis of $aadB$ copy number.** Genomic DNA samples (2 µg) from P. stutzeri Q derivatives were digested for 3 h with 20 U EcoRI, EcoHI, NcoI, PstI, SalI, SmaI or SstI and run on 0.8% agarose gels. Southern blotting was done by capillary transfer in 20 x SSC buffer (Sambrook & Russell, 2001) onto positive nylon membranes (Hybond-N+; Amershams Biotec) with subsequent hybridization at high stringency (68°C) according to the DIG kit instructions (Roche). The probe consisted of the DIG-dUTP-labelled $aadB$ gene (573 bp), which was PCR-amplified (primers NVC33/NVC34) from a gel-purified PstI-KpnI fragment of pUS23. The CDP-Star reagent (Roche Applied Science) was used for detection.

**Cloning and recovery of integrated reporter plasmid.** Based on hybridization with $aadB$, a 12 kb Std genomic fragment from strain Q23-17 was excised from agarose, purified, ligated to EcoRV-digested, alkaline-phosphatase-treated pACYC184 and electroporated into JM109. Resultant Cm$^R$ colonies were screened for loss of Tc$^R$ by patching to LB-Cm and LB-Tc plates, then Tc$^R$ clones were screened by PCR using NVC70/NVC71 to detect attI-$aadB$ junctions. One positive clone was retained and designated pUS40. Plasmids were extracted from a 200 ml LB-Cm-Ap culture of E. coli JM109(pUS40) and used for sequencing with pUS23 construction primers, pUC19 vector primers and P. stutzeri Q integron primers (Table 2).

**Analysis of Ap$^R$ plasmids in P. stutzeri Q derivatives.** Total genomic DNA was extracted from the Ap$^R$ Gm$^R$ P. stutzeri Q(pUS23) strains Q23-7, Q23-17 and Q23-25, and the parental Ap$^R$ P. stutzeri Q(pUS23) strain. DNA from each strain (10 µg) was electroporated into JM109 and, after recovery in LB broth, the cells were plated on either Ap or Gm medium. The mean Ap$^R$ and Gm$^R$ transformation frequencies were calculated from three experiments. From each set of Ap$^R$ JM109 transformants, five clones were subcultured in LB-Ap broth, and the plasmids extracted and analysed by restriction-mapping (EcoRI/HindIII digest). An insertion in one plasmid was further analysed by sequencing using primers NVC113/NVC114.

**Detection of $aadB$ expression by RT-PCR.** RNA was extracted from late-exponential-phase cultures of P. stutzeri strain Q23-17 using the RNeasy kit (Qiagen) according to the manufacturer’s instructions, with the following modifications. Approximately $2 \times 10^7$ cells (2 ml culture at OD$_{600}=1$·0) were pelleted from broth and resuspended directly in buffer RLT, omitting lysozyme treatment. DNase incubation (37°C, 30 min) was done with the RNA eluate rather than on-column and was followed by a second round of column purification. cDNA was prepared with Omniscript RTase (Qiagen) using 1 μg RNA and primer NVC108. Subsequent PCR reactions used 1 μl RT mixture with primers NVC91/NVC71 and A/H21/NVC71, and were performed under the same conditions as other PCRs (above). Negative controls contained either no RT enzyme or DNA from wild-type P. stutzeri Q, while a positive control contained DNA from strain Q23-17.

**RESULTS**

**Construction and characterization of reporter pUS23 in E. coli**

The reporter plasmid pUS23 (Fig. 1) mimics a gene cassette in that it is a circular element and includes promoterless genes ($aadB$ Gm resistance and $gfpmut3$ green fluorescence) immediately downstream of a 59-bp recombination site ($aadB$ 59-bp). It is distinct from naturally occurring gene cassettes in that it contains a plasmid replication origin, a resistance gene (Ap$^R$) with its own promoter and transcription terminators ($rrnBT1T2$) upstream of the $aadB$ 59-bp. Addition of the terminators was found to be necessary to ensure silence of the Gm$^R$ and green fluorescence genes in JM109 (data not shown). In E. coli, pUS23 is replicative and has the phenotypic Ap$^R$ Gm$^R$ GFP$^-$ (Table 1). The sequence of pUS23 has been deposited in GenBank (AY894753). The narrow host range of PUC vectors means pUS23 is expected to be non-replicative in most bacteria. In cells containing functional integrons, capture of pUS23 could potentially give Ap resistance, Gm resistance and green fluorescence. In the case of Ap$^R$, this phenotype could result from integron-mediated recombination alone (at attI, 59-bp or a secondary site), while in the cases of Gm$^R$ and green fluorescence, the phenotypes require both recombination (at attI) and expression (from $P_c$).

**Activation of cassette-associated phenotypes in P. stutzeri cells**

Three criteria are necessary for activation of gcORFs. These are uptake of a cassette by transformation, capture of the cassette by a replicon within the cell and transcriptional activation of the gcORFs. Electroporation of pUS23 into P. stutzeri Q resulted in an unexpectedly high number of colonies on Ap plates [mean 6·2 x 10$^4$ c.f.u. (µg plasmid)$^{-1}$ over two experiments], but none on Gm plates. Since individual colonies from Ap plates were unable to be subcultured onto LB-Ap plates it is probable this does not reflect chromosomal integration of the plasmid (e.g. at attIPstQ). When DNA was extracted from the total pool of P. stutzeri Q(pUS23) transformant colonies, plasmid bands were not visible in agarose gels (data not shown), but electroporation...
of this DNA into *E. coli* JM109 did yield Ap<sup>R</sup> JM109 transformants at low frequencies [approx. 20 c.f.u. (µg DNA)<sup>-1</sup>]. A possible explanation is that the founder cells for the Ap<sup>R</sup> colonies were those in which pUS23 was initially present, but not effectively maintained during further growth of the colonies. We subsequently found one report of pUC vector maintenance in a *P. stutzeri* strain that provides support for this hypothesis (Pemberton & Penfold 1992).

Whilst high levels of initial transformation to Ap<sup>R</sup> were observed, the lack of activation of pUS23 reporter phenotypes suggests that either the wild-type integron is not active under the laboratory growth conditions or the frequency of recombination was below the transformation frequencies attainable in our experiments. In previous reports on integron activity, the frequencies of cassette activation by wild-type class I integrons were low (10<sup>-7–10</sup><sup>-8</sup>) (Hall *et al*., 1991; Recchia *et al*., 1994), but still well above the level of spontaneous Gm<sup>R</sup> observed here (<10<sup>-10</sup> per cell). We experimented with a number of variables aimed at increasing the likelihood of observing recombination events, including increasing the plasmid concentration and extending the time available for recombination to occur (Collis *et al*., 1993). A two-step approach, in which pooled Ap<sup>R</sup> *P. stutzeri* Q(pUS23) transformants were subcultured in LB-Ap broth prior to plating out on to LB-Gm media was ultimately successful in yielding Gm<sup>R</sup> colonies. In these experiments Gm<sup>R</sup> colonies arose from the LB-Ap subculture at a frequency of 2·5 × 10<sup>-7</sup> per c.f.u. (mean of three experiments).

At least three broad categories of event capable of activating *aadB* expression can be postulated; integron-related events where IntIPstQ mediates integration of pUS23 at attIPstQ and a *Pc* promoter directs expression of the genes; other chromosomal integration events where pUS23 is integrated downstream of a chromosomal promoter either by IntIPstQ or by other recombinases; and plasmid-related events whereby mutation or rearrangement of the plasmid leads to enhanced maintenance of the plasmid and activation of reporter genes.

**Capture of pUS23 by InPstQ occurred by site-specific recombination**

We expected that the majority of reporter gene activation events would be integron-related. Accordingly, PCR screening targeted the attI-aadB junction predicted to be formed as a result of integration of pUS23 at attIPstQ. Approximately 30 Gm<sup>R</sup> colonies from each of three independent transformation experiments were screened and products of the expected size (425 bp) were found in 4/32, 2/23 and 1/27 cases. One PCR-positive strain from each experiment (Q23-7, Q23-17 and Q23-25; hereafter referred to as attI-Gm<sup>R</sup> strains) was further tested by PCR-targeting the predicted *aadB* 59-be/BGC001 junction between InPstQ and pUS23, and a product of the expected size (509 bp) was observed (Fig. 2). The junction PCR products from the attI-Gm<sup>R</sup> strains were sequenced and aligned to the known pUS23 and InPstQ sequences (Fig. 3). Crossovers located within the 1R core site (GT TAGGC) of the *aadB* 59-be (Stokes *et al*., 1997) and the comparable core site of attIPstQ (also GT TAGGC) were observed in all cases, indicating that the reporter plasmid had integrated via site-specific recombination, most likely mediated by IntIPstQ. Therefore, on average, 8% of all Gm<sup>R</sup> colonies contained pUS23 integrated at attIPstQ. Based on the frequency of appearance of all types of Gm<sup>R</sup> colonies (2·5 × 10<sup>-7</sup> per c.f.u.; see above), the overall frequency of attI integration can be calculated to be 2·0 × 10<sup>-8</sup> per cell.

The phenotype of the attI-Gm<sup>R</sup> strains was further investigated. The MIC for Gm ranged from 15 to 20 µg ml<sup>-1</sup>, while the MIC for wild-type *P. stutzeri* Q was 3 µg ml<sup>-1</sup>. All attI-Gm<sup>R</sup> strains also showed strong Ap<sup>R</sup> (MIC > 100 µg ml<sup>-1</sup>), and both resistances were stably maintained after at least one round of subculture on antibiotic-free medium, in contrast to the unstable Ap<sup>R</sup> of the initial *P. stutzeri* Q(pUS23) transformants (data not shown). All the attI-Gm<sup>R</sup> strains were green-fluorescent by microscopy (Fig. 2), confirming that both reporter genes had been activated and that Gm<sup>R</sup> was unlikely to be due to a spontaneous chromosomal mutation. The stabilization of Ap<sup>R</sup>, and the appearance of Gm<sup>R</sup> and fluorescence are all consistent with integron capture and expression of the pUS23 gene cassette.

**Detection of aadB copies by Southern hybridization**

Since a large proportion of Gm<sup>R</sup> strains did not involve integration of pUS23 at attIPstQ, we tested for the possibility
of additional pUS23 integration events in the attI-GmR strains. Genomic DNA from the three attI-GmR strains was extracted, digested with PstI and Southern blots were probed with the aadB gene (Fig. 4). A unique PstI site is situated upstream of aadB in the reporter plasmid and a PstI site is found in the third P. stutzeri Q gene cassette (BGCO03), and thus we expected to observe a band of 5-5 kb representing attI-integrated pUS23, and possibly also a band at 4-3 kb representing free plasmid. The hybridization pattern of strain Q23-7 was consistent with the presence of both free and attI-integrated pUS23 (Fig. 4). Strain Q23-25 was also predicted to contain free pUS23 (4-3 kb band), but an inferred attI-integrated band was at 6-6 kb rather than the expected 5-5 kb.

In strain Q23-17 the only band detected was an inferred attI-integrated copy, but again this was at 6-6 kb, rather than the 5-5 kb predicted for the original pUS23. Only one copy of aadB was present in strain Q23-17 based on Southern hybridizations with DNA digested with a wide range of enzymes (Fig. 4b), but bands observed in the digests were in almost all cases of different sizes to those predicted from the pUS23 and InPstQ sequences. In the absence of digestion, a hybridization signal was seen with chromosomal DNA, consistent with chromosomal integration rather than free reporter plasmid. Taken together with the junction sequencing data presented above, the source of the GmR and green fluorescence phenotypes in strain Q23-17 appeared to be a derivative form of the pUS23 gene cassette with an approximately 1 kb insertion integrated at the attI site of InPstQ.

**Sequence analysis of integrated pUS23 and flanking integron DNA from strain Q23-17**

Due to the unexpected complexity of the Southern blot data and the implied presence of pUS23 and derivative plasmids in both free and integrated forms, questions relating to pUS23 structure, genomic location and reporter gene expression remained. To address these questions, we cloned and sequenced a 12 kb Stul fragment of strain Q23-17 DNA containing pUS23 with flanking chromosomal DNA on either side (pUS40). Extensive sequencing of the Stul fragment confirmed the integration of a derivative form of the pUS23 gene cassette by site-specific recombination at an attI site. Through PCR, partial sequence analysis and restriction mapping of pUS40, this site was confirmed to be in the previously described integron InPstQ (Holmes et al., 2003b). The flanking sequences of the Stul insert included the expected 167 bp of intIPstQ at one end and 586 bp of the P. stutzeri Q chromosomal gene orf136 at the other end of the insert DNA. PCR and restriction mapping indicated that the 10 gene cassettes of the wild-type InPstQ array were present in the Stul fragment in their original order (data not shown).

The near-complete sequence of the integrated pUS23 derivative in the cloned Stul fragment was obtained and the only sequence variation relative to the initial construct, pUS23, was the presence of an insertion sequence (IS) between bla and ori, inserted at TTAAGGATCTAGG-TGAAG. The 1191 bp IS element in the integrated pUS23 was designated ISPst5 (GenBank AY894752) and is a
member of the IS5 family. ISPst5 encodes a 326 aa DDE-type transposase, has 12 bp perfect terminal inverted repeats and creates a 4 bp target repeat (CTAG). ISPst5 has 99% DNA identity to an IS associated with alkylbenzene degradation genes in Pseudomonas putida 01G3 (Chablain et al., 2001) and 83–86% DNA identity to IS elements associated with naphthalene and carbazole catabolic plasmids in other Pseudomonas strains (Dennis & Zylstra, 2004; Nojiri et al., 2004). Since IS elements may have pleiotropic effects via promoters reading out from the IS (Galas & Chandler, 1989), we examined in more detail the pleiotropic effects via promoters reading out from the IS that GmR and green fluorescence in this strain were due to an IS associated with naphthalene and carbazole catabolic degradation genes in Pseudomonas putida 01G3 (Chablain et al., 2001) and 83–86% DNA identity to IS elements associated with alkylbenzene degradation genes in other Pseudomonas strains (Dennis & Zylstra, 2004; Nojiri et al., 2004). Since IS elements may have pleiotropic effects via promoters reading out from the IS (Galas & Chandler, 1989), we examined in more detail the expression of reporter genes in the attl-GmR strain Q23-17.

Expression of aadB and gfp is from a Pc equivalent promoter in InPstQ

The orientation of attl-integrated pUS23 in strain Q23-17 was as shown in Fig. 1(c) and provided good initial evidence that GmR and green fluorescence in this strain were due to an IS associated with naphthalene and carbazole catabolic degradation genes in Pseudomonas putida 01G3 (Chablain et al., 2001) and 83–86% DNA identity to IS elements associated with alkylbenzene degradation genes in other Pseudomonas strains (Dennis & Zylstra, 2004; Nojiri et al., 2004). Since IS elements may have pleiotropic effects via promoters reading out from the IS (Galas & Chandler, 1989), we examined in more detail the expression of reporter genes in the attl-GmR strain Q23-17.

Characterization of original and derivative pUS23 plasmids in GmR P. stutzeri Q strains

Based on hybridization data (Fig. 4), two of the three attl-GmR strains appeared to contain free plasmid. While strong evidence was obtained that integration of pUS23 at attl was responsible for the GmR phenotype of Q23-17, it was possible that plasmid rearrangements, mutations or insertion sequences contributed to GmR in the other two attl-GmR strains. To examine this possibility, genomic DNA from the attl-GmR strains was electrophorated into E. coli JM109. These experiments yielded ApR colonies from Q23-7 [49±13 c.f.u. (μg DNA)⁻¹] and Q23-25 [52±21 c.f.u. (μg DNA)⁻¹], but not from Q23-17, consistent with the Southern hybridization data (Fig. 4). None of the experiments yielded GmR transformants of E. coli.

Restriction mapping of five recovered plasmid clones from Q23-7 and Q23-25 indicated an insertion of 1.2 kb in one plasmid from Q23-25, while all the other plasmids matched the profile of the original pUS23 (data not shown). Sequencing revealed that the insertion was ISPst5, in an identical location to that seen in the attl-integrated pUS23 copy in strain Q23-17 (between bla and ori in the reporter backbone). The fact that two independent isolates contained ISPst5 inserted at identical plasmid locations suggests a

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**Fig. 5.** Localization of Pc promoter by RT-PCR analysis of aadB gene expression in strain Q23-17. (a) Schematic diagram of attl region of InPstQ. Primer binding sites and restriction sites are underlined, while promoter elements (−35 and −10 regions), the interPstQ start codon, the attlPstQ-aadB junction (GTATTGGC), the aadB ribosome-binding site (RBS) and start codon are shown in bold. The binding site of reverse primer NVC71 (not shown) is approximately 200 bp downstream of the aadB start codon. (b) RT-PCR data showing amplification with RNA and DNA templates from strain Q23-17 (shown as ‘17’) and P. stutzeri Q (shown as ‘Q’) using two alternative primer pairs. Reactions with and without reverse transcriptase are designated +RT and −RT, respectively.
degree of target specificity for this insertion sequence, which is consistent with data available for IS5 family IS elements, which preferentially integrate at the sequence YTar (usually CTAG, as observed with ISPst5) (Mahillon & Chandler, 1998). None of the E. coli JM109 transformants harbouring recovered pUS23, or the derivative pUS23-ISPst5, were GmR. Retransformation of the pUS23-ISPst5 plasmid from Q23-25 back to wild-type P. stutzeri Q confirmed that this plasmid conferred ApR, but not GmR, indicating that changes in the reporter plasmid itself were not responsible for GmR and that the activation of the cassette-associated genes occurred via the CI.

**DISCUSSION**

### The significance of CIs

The gene cassette metagenome is a major source of genetic novelty. Through their manipulation of this resource, integrons are expected to have significant evolutionary impact. Based on the class 1 integron paradigm, where diverse antibiotic resistance genes were assembled in less than 50 years, natural selection for advantageous combinations of genes can occur relatively quickly. CIs are particularly interesting in this regard, since a particular integron backbone may operate within a bacterial lineage over evolutionarily significant periods of time (Rowe-Magnus et al., 2003; Gillings et al., 2005). It is plausible that CIs have been continually acquiring gene cassettes, expressing their associated genes and rearranging their transcriptional order throughout the evolution of certain genera. In the cases of Vibrio and Xanthomonas, CIs are inferred to have been resident over thousands of years, ample time for selection of beneficial gene combinations. But do CIs have the same capabilities as mobilized integrons? MIs show high diversity in 59-be sites and their associated gcORFs have a tendency to exhibit a functional theme. It seems reasonable to infer that the apparent linkage between gcORF function and 59-be diversity reflects natural selection for gene cassettes from different sources that deliver related fitness advantages. In contrast, CIs share similarity of 59-be sites within a species and it seems unlikely that cassettes in CI arrays, particularly very large ones, share a functional theme. The fixed chromosomal location of CIs is expected to limit their access to diverse sources of gene cassettes, but do these patterns in CI and MI cassette arrays also reflect different abilities to facilitate natural selection for useful combinations of gene cassettes?

Two possibilities worthy of investigation are that not all integrons may have the full set of functions observed in resistance integrons, or that all integrons have the same functions but differ in their level of activity. For example limitation or absence of gcORF expression would largely remove the selective mechanisms capable of leading to accumulation of gene cassettes from different backgrounds (containing diverse 59-be) or with functionally related phenotypes. Alternatively, higher recombination specificity and/or lower frequencies of recombination activity could also explain why CIs are not associated with readily detectable phenotypes since the chance of acquiring a selectively advantageous gene would be reduced. In summary two key questions for CIs are: do they express gcORFs, and is the rate and/or specificity of gene cassette capture limited with respect to the mobilized integrons? Answering these questions requires better sampling of integron diversity and methods to look at integron activity in a natural context. Here we have used a reporter gene cassette to address these questions.

### The InPstQ CI can activate gene cassettes

Our data are the first demonstration that native-form CIs can capture gene cassettes and express cassette-associated genes, and greatly extend previous observations that CIs can act as accessible reservoirs of gene cassettes (Rowe-Magnus et al., 2002). Cloning, sequencing, hybridization and RT-PCR data confirmed that integron activity resulted in gcORF expression. Hybridization and sequence data showed that in three independent strains pUS23 was captured by site-specific recombination between attlPstQ and the aadB 59-be of pUS23. In strain Q23-17 subsequent activation of the aadB and gfp genes was shown to be due to expression from a promoter located within InPstQ. We conclude that InPstQ is a fully functional integron whose activities are sufficient to enable the capture of gene cassettes and expression of associated genes. While we cannot extrapolate from our study of InPstQ to infer functions of other CIs, it should be possible to use pUS23 or similar reporter gene cassettes to rapidly screen other CIs for function.

Interpretation of the data was complicated by the presence of an insertion sequence in the reporter cassette. Examples of both original and IS-derivative pUS23 were rescued by transformation into E. coli JM109. The derivative form was shown to have a GmR GFP+ phenotype in E. coli and when transformed back into wild-type P. stutzeri. These data show that ISPst5 does not cause the GmR GFP+ phenotype. The association between ISPst5 and pUS23 is also highly unlikely to be a prerequisite for capture of the pUS23 cassette by InPstQ, since one strain (Q23-7) appeared to contain only the original pUS23. However, the interaction between ISPst5 and pUS23 may have had other effects (see below).

### Interaction between pUS23 and ISPst5 is likely to have influenced the frequency of cassette capture

The other outstanding issues with respect to CI function are the rate and specificity of gene cassette capture. With respect to 59-be specificity, it is notable that InPstQ was able to recognize the aadB 59-be, a recombination site that is divergent from the 59-be family typically observed in Pseudomonas CI arrays (Holmes et al., 2003b; Vaisvila et al., 2001). We did not investigate this further here. Our estimate (2 × 10^-8 per cell) of the frequency of cassette capture and activation by InPstQ is of a similar order of magnitude to

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frequencies of cassette phenotype activation observed with wild-type class I integrons (Hall et al., 1991; Recchia et al., 1994). However, these numbers are not directly comparable due to different methodologies. In addition, the effect of experimental conditions such as antibiotic exposure is unknown (Beaber et al., 2004) and the data are further complicated by the fact that pUS23 did not behave as a non-replicative gene cassette in our system.

P. stutzeri is an exceedingly diverse species complex (Cladera et al., 2004) and there are reports of CoIE1-like replicons being replicative in some strains and non-replicative in others (Pemberton & Penfold, 1992). Here, pUS23 was found to be maintained to some extent in P. stutzeri Q. It is probable that this increased the chance of recombination between the aadB 59-be and attIPstQ, thus increasing the frequency of observation of cassette capture. Surprisingly, this effect was apparently exacerbated by ISPst5 insertion. The finding that two independently isolated pUS23 plasmid derivatives contained ISPst5 inserted upstream of ori in the pUC19 backbone raises the possibility that the insertion may have stimulated the origin of replication, which was initially only weakly functional in P. stutzeri Q. Activation of the pBR322 plasmid origin by an IS-derived promoter has been observed with Tn5 (Lupski et al., 1986). In any event the maintenance of the cassette as a plasmid allowed us to detect low frequency events that may otherwise have not been observed with a non-replicative cassette.

Are there alternate routes for gene cassette activation?

It is widely thought that integrons are the major route by which cells access the gene cassette metagenome. A surprising outcome of our study was that less than 10% of GmR P. stutzeri Q colonies resulted from integration at attIPstQ (we term the remainder unknown-GmR strains). Southern hybridization revealed that pUS23 was at heterogeneous genomic locations in three representative unknown-GmR strains (data not shown), suggesting that multiple routes for pUS23 reporter activation exist. These could include alternate integron sites or recombination involving insertion sequences.

It is unlikely that pUS23 initially contained sequences that could support homologous recombination with the P. stutzeri chromosome, and Southern blots (Fig. 4) confirmed that aadB was absent from wild-type P. stutzeri Q. However, insertion of ISPst5 into pUS23 would provide a convenient target for homologous recombination with other chromosomally borne copies of the same IS. Similar reactions are believed to be responsible for the integration of the F plasmid into the E. coli chromosome and subsequent production of Hfr strains (Galas & Chandler, 1989). In the case of the unknown-GmR strains such events would also need to have resulted in expression of aadB and gfp.

Integrons show a strong preference for capture of gene cassettes by recombination at their cognate attI site (Collis et al., 2002b), although cassette capture by recombination at 59-be sites or secondary sites (not associated with integrons or gene cassettes) is known (Francia et al., 1993; Recchia et al., 1994) and expression from promoters upstream of secondary sites has been demonstrated (Segal & Elisha, 1999). We screened three of the unknown-GmR strains (Q23-10, Q23-12 and Q23-13) for insertion of pUS23 at the 59-be sites associated with the 10 gene cassettes in the InPstQ array by PCR (data not shown), but the results indicated that pUS23 had not integrated into the known cassette array. Another possibility is that attI, 59-be or secondary sites may exist elsewhere in the chromosome. For example, Pseudomonas alcaligenes harbours multiple cassette arrays (Vaisvila et al., 2001), and the genomes of Shewanella (GenBank NC_004347) and Treponema (GenBank NC_002967) contain gene cassettes at locations isolated from the known integron. IntI-mediated integration of gene cassettes at secondary sites has been reported. If alternate attI or 59-be sites were the site of integration, then we must also account for the low proportion of capture at the attI site of InPstQ, as previous studies have suggested the cognate attI site is strongly preferred over 59-be sites (Collis et al., 2002a, b).

The location of Pc is worth noting in the context of integration site preference. The implied overlap of intI and cassette transcripts suggests the potential for a regulatory relationship between expression of these two integron elements. This makes ‘biological sense’ in that if a cell is expressing an advantageous combination of gcORFs, concomitant IntI activity would potentially result in disruption. Previous studies on recombination site preferences have all involved assays where the integrase was supplied in trans and not under the control of its native promoter, while in our system the native integron was assayed for function. If there is a regulatory relationship between intI expression and Pc-directed gcORF expression then this could influence the observed outcomes. This is worthy of further investigation.

The pUS23 reporter as a tool for integron recovery

Since the gene cassette metagenome is tightly linked to the activity of integrons, understanding integron distribution and diversity is a key element in determining the evolutionary significance of gene cassettes. In this context, another application for the pUS23 reporter cassette is in the recovery of new integrons. Screening for resistance phenotypes clearly does not recover representative integron diversity (Barlow et al., 2004) and PCR-based screening strategies (Nemerugut et al., 2004; Nield et al., 2001) are limited to recovery of integron fragments. Useful features for second-generation reporter constructs may include broad host-range (Kovach et al., 1995) or conditional (Herrero et al., 1990) replication origins, and mobilization functions compatible with promiscuous E. coli delivery vehicles (Simon et al., 1983). A reporter-based screening strategy would have the advantage of detecting functional
integrons and allow recovery of the intact integron in its native host. Application of this approach to environmental samples could yield valuable information on the in situ activity and ecological relevance of various integron types.

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REFERENCES


