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ORIGINAL ARTICLE

Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment

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The impact of human activity on the selection for antibiotic resistance in the environment is largely unknown, although considerable amounts of antibiotics are introduced through domestic wastewater and farm animal waste. Selection for resistance may occur by exposure to antibiotic residues or by co-selection for mobile genetic elements (MGEs) which carry genes of varying activity. Class 1 integrons are genetic elements that carry antibiotic and quaternary ammonium compound (QAC) resistance genes that confer resistance to detergents and biocides. This study aimed to investigate the prevalence and diversity of class 1 integron and integron-associated QAC resistance genes in bacteria associated with industrial waste, sewage sludge and pig slurry. We show that prevalence of class 1 integrons is higher in bacteria exposed to detergents and/or antibiotic residues, specifically in sewage sludge and pig slurry compared with agricultural soils to which these waste products are amended. We also show that QAC resistance genes are more prevalent in the presence of detergents. Studies of class 1 integron prevalence in sewage sludge amended soil showed measurable differences compared with controls. Insertion sequence elements were discovered in integrons from QAC contaminated sediment, acting as powerful promoters likely to upregulate cassette gene expression. On the basis of this data, $>1 \times 10^{19}$ bacteria carrying class 1 integrons enter the United Kingdom environment by disposal of sewage sludge each year.
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Introduction

Horizontal gene transfer is responsible for disseminating a large range of antibiotic resistance genes on mobile genetic elements (MGEs) between disparate groups of bacteria. Resistance gene transfer occurs not only within the clinic, but in the natural environment (Gaze *et al.*, 2008). The acquisition of resistance genes by human pathogens from environmental bacteria has been demonstrated in several cases, such as the extended spectrum β-lactamases, CTX-M genes, which originated in environmental bacteria (Poirel *et al.*, 2002; Olson *et al.*, 2005; Hawkey, 2008; Nordmann *et al.*, 2008; Rossolini *et al.*, 2008). However, the role of the environment in driving the development of antibiotic resistance in the clinic is still a matter of controversy.

Important genetic elements involved in the development of resistance are integrons, particularly class 1 integrons. These elements carry an integrase gene, which controls integration and excision of genes from a mobile cassette gene metagenome (Mazel, 2006). Most cassettes of known function carried by class 1 integrons confer antibiotic or quaternary ammonium compound (QAC) resistance, although there is increasing evidence of additional adaptive genes encoded by cassette genes (Koenig *et al.*, 2009). QACs are used widely as detergents and disinfectants in domestic, industrial and clinical settings. It is clear that integron-associated genes are capable of conferring resistance to a wide range of antibiotics, including extended spectrum β-lactams, carbapenems and fluoroquinolones, representing an extremely efficient method of acquiring resistance to the most widely used and important clinical antibiotics (Fluit and Schmitz, 2004).

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The existence of environmental reservoirs of class 1 integrons has been known for some time (Rosser and Young, 1999), and the fact that these elements are able to carry antibiotic and biocide resistance genes led to the idea that certain widely distributed pollutants such as QACs may co-select for antibiotic resistance by selecting for class 1 integrons themselves (Russell, 1999). Only in recent years has evidence emerged of a link between biocide and antibiotic resistance, with most earlier work finding little or no data to suggest co-selection (Russell, 2002). The first study to establish a correlation between environmental QAC exposure and increased prevalence of class 1 integrons investigated bacterial populations in sediment from an engineered wetland or reed bed system used to remediate effluent from a textile factory with high QAC usage. QACs were used as scouring agents, antistatic agents (used at 0.1–0.2% of the fabric weight), dyeing retardants, softeners, finishers and moth-proofers (Gaze *et al.*, 2005). Culturable bacterial populations were highly resistant to ditallowdimethylammomium chloride and cetyltrimethylammonium bromide compared with control populations from agricultural soils, and class 1 integron prevalence was significantly higher in reed bed isolates. More than 95% of integron carrying isolates possessed the *qacE* gene encoding QAC resistance (Gaze *et al.*, 2005). More recent work investigating *qac* gene carriage in environmental bacteria illustrated a diverse range of class 1 integron-borne genes in bacteria from water samples collected in the metropolitan Sydney area (Gillings *et al.*, 2009b), including *qacE*, *qacG* and *qacH*. Prevalence of *qac* genes was reported as being approximately half that of *intI1*. The latter authors investigated the presence of the three clades of genes as being in the form of gene cassettes by comparing internal amplicons from *qacE*, *qacG* and *qacH* with an amplicon including part of the cassette attC site, concluding that banding patterns were the same and, therefore, the genes were all integron borne cassettes. Many class 1 integrons also carry *qacEΔ1*, which is a functional deletion of *qacE* and is found adjacent to *sul1* encoding sulphonamide resistance in the 3′-conserved segment of some class 1 integrons (Paulsen *et al.*, 1993) and is characteristic of class 1 integrons found in the clinic (Stokes and Hall, 1989). Other integron-borne *qac* genes reported in the literature include *qacF*, *qacI* (synonymous with *qacH*) and *qacK* (Partridge *et al.*, 2009).

Bacteria bearing class 1 integrons have been reported in the wastewater system (Ghosh *et al.*, 2009) and in farm slurries spread to agricultural land (Heuer and Smalla, 2007; Byrne-Bailey *et al.*, 2009, 2010), however, the impact of sewage sludge and farm slurry amendment to land on environmental reservoirs of class 1 integrons is poorly studied.

Our aim was to investigate the impact of biocide and antibiotic residues on integron and cassette gene prevalence in environmental samples (class 1 integrons and QAC resistance gene cassettes), with sample site selection designed to unravel the two selective pressures. QAC polluted samples were taken from a reed bed used to remediate effluent from a textile mill (Gaze *et al.*, 2005), and antibiotic selection was investigated in slurry from tylosin fed pigs, experimentally amended with antibiotics. An intermediate scenario with bacterial populations encountering QAC and antibiotic residues was represented by samples taken from anaerobic digesters in a wastewater treatment plant. These samples were also taken to assess integron carriage in fully digested sewage sludge disposed of to land. A sample site consisting of fallowed agricultural soil with no history of sewage sludge or animal slurry amendment was selected as a control. Multiple samples were also taken from land amended with sewage sludge 1, 12 and 24 months after application. We examined class 1 integron and QAC resistance gene abundance using real-time PCR, and novel integron promoter modifications.

Materials and methods

Sampling

Cores were taken from a reed bed used to remediate effluent from a textile mill with high usage of QACs but with no sewage or antibiotic content (two cores taken 2 m apart in two separate sections of the reed bed). Treated sewage sludge was sampled from four anaerobic digesters at a wastewater treatment plant containing QACs from wastewater and antibiotic residues. Pig slurry was obtained from tylosin fed pigs and amended with oxytetracycline and sulfachloropyridazine at concentrations of 25.58 mg l^{-1} and 18.85 mg l^{-1}, respectively, which are representative of actual concentrations found in slurry (four samples taken from slurry tank); this was part of a long-term study to elucidate the environmental fate of veterinary antibiotics (Boxall *et al.*, 2002). Cotswold soil was from a farm with no known history of sludge or slurry amendment (four cores taken at >10 m intervals). Further sampling was undertaken at a farm where conventionally treated, limed and dewatered sewage sludge was added at 240 kg per hectare, 10 cores (10 cm cores, taken >10 m apart, mixed before sub-sample removed for DNA extraction) were taken from each treatment plot including plots amended 1, 12 and 24 months previously, and one control plot. Four replicate samples of limed and dewatered sewage cake added to farm plots were also analysed.

DNA extraction

Template DNAs were extracted from environmental samples using the UltraClean Soil DNA Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions; 0.4 g samples were used.

Real-time PCR

In each 40 μl reaction, there were 20 μl 2 × Power Sybr Green PCR Master Mix (Applied Biosystems,

Foster City, CA, USA), 4 μl of primer pairs, 0.4 μl bovine serum albumin (10 mg ml^{-1}), 4 μl of 10 times diluted template DNA and 11.6 μl DNA free H_2O. Final concentration of primers pairs were 0.9 μM for 16S1369f/1492r (Nandi *et al.*, 2004), int1f2/int1r2 and qacEfor/qacErev and 0.45 μM for qacEcom1/qacEcom1rev (for primer sequences, see Table 1). *qacEΔ1* numbers were determined by subtracting the number of *qacE* amplicons from the number of amplicons generated using primers to a region common to both *qacE* and *qacEΔ1*. The primers were designed using Primer Express 3.0 for Real Time PCR Program (Applied Biosystems). PCR cycle conditions in stage 1, 50 °C 2 min, then 95 °C 10 min, for one cycle; in stage 2, 95 °C 20 s then 60 °C 1 min, for 40 cycles. The dissociation curve step was added as stage 3. The reactions were carried out using an Applied Biosystems 7500 Fast System. Standard curves for absolute quantification of *intI1*, *qacE* and *qacEΔ1* were produced from serial dilutions of *E. coli* SK4903 (IncPβ R751 carrying *intI1*, *qacE*) (Paulsen *et al.*, 1993) enumerated by viable plate counts and *qacG* and *qacH* from PCR products enumerated by spectrophotometry. *qacE* was enumerated from standard curves generated by both methods (36 PCRs) to correct for possible biases introduced by standard curve construction. Molecular prevalence was calculated by dividing the number of target genes by the number of 16S rRNA copies, with corrections made for 16S rRNA (seven in *E. coli* in seeded standards, mean 2.5 copies per genome in all bacteria) and IncPβ R751 copy number (average six copies per cell in seeded standards) (Adamczyk and Jagura-Burdzy, 2003; Acinas *et al.*, 2004). Specificity of all real-time PCRs was confirmed by sequencing PCR products. Several dilutions of template DNA were used for each sample to ensure the effect of PCR inhibitors was accounted for.

Construction of gene cassette libraries

Primer pair cass1/cass2 (Rosser and Young, 1999) and PCR Master Mix (Promega, Madison, WI, USA) were used to amplify class 1 integron fragments flanked by *intI1*and *qacE/qacEΔ1* from environmental DNAs. PCR fragments were cleaned up with PCR Clean Kit (Qiagen, Gaithersburg, MD, USA), ligated to pGemT-easy vector (Promega) and the ligation mixtures transformed to JM109 *E. coli* cells (Promega). Qiaprep Spin Miniprep Kit (Qiagen) was used to purify the recombinant plasmids. The SP6 primer was used for sequencing the cloned inserts.

*Construction of the promoter–*lacZ *transcriptional fusion plasmids*

Putative promoter fragments were PCR-amplified with either restriction site *Eco*RI or *Bam*HI added to the 5′-end and with either *Bam*HI or *Hind*III site added to the 3′-end (see Supplementary Table S1 for primer sequences used in cloning). Promoters cloned into the *lacZ* reporter vector pRW50 (Lodge *et al.*, 1992) were integron cassette Pc promoter, Pc promoter plus putative *qacE* promoter, putative *qacE* promoter, putative fusion promoter provided by ISPpu*17* and *qacE*, putative promoter provided by ISUnCu*13* plus *qacE* promoter, the *pBad* promoter (positive control) and part of the *araC* gene coding sequence from the pBad24 plasmid (negative control). PCR fragments were digested with corresponding *Eco*RI plus *Hind*III or *Eco*RI plus *Bam*HI or *Bam*HI plus *Hind*III to produce cohesive ends. The same combinations of restriction enzymes were used to digest the promoter-less *lacZ* reporter vector pRW50. The digested PCR fragments were ligated into the vector and transformed into *E. coli* JM 109 cells. Tetracycline-resistant transformants were selected and those containing plasmids were prepared using the Qiagen plasmid mini-prep kit. The promoter fragments in the plasmids were sequenced using the primer prw50f to confirm the correct sequence. The confirmed plasmids were transformed into *lacZ*-negative *E. coli* strain M182.

β-galactosidase activity assay

The *E. coli* M182 strains harbouring various recombinant plasmids were grown in lysogeny broth with

Table 1 PCR primers and their targets used in this study

Name of primers	*Target*	*Sequence 5′–3′*	*References*
16S1369f	16S rRNA	CGGTGAATACGTTCYCGG	Nandi *et al.* (2004)
16S1492r	16S rRNA	GGWTACCTTGTTACGACTT	Nandi *et al.* (2004)
cass1	*intI1*	TGATGCGCATGCCCGTTCCATACAG	Rosser and Young (1999)
cass2	*qacE/qacEΔ1*	GGCAAGCTTAGTAAAGCCCTCGCTAG	Rosser and Young (1999)
Int1F2	*intI1*	TCGTGCGTCGCCATCACA	This study
Int1R2	*intI1*	GCTTGTTCTACGGCACGTTTGA	This study
QacEfor	*qacEΔ1*	CTTCATGGGCAAAAGCTTGATG	This study
QacErev	*qacEΔ1*	TTAGTGGGCACTTGCTTTGGA	This study
QacEcom1f	*qacE*	GTTATGGCATCGCATTTTATTTTCT	This study
QacEcom1r	*qacE*	CCGACCAGACTGCATAAGCA	This study
QacGf2	*qacG*	TGTCGCTGACACTCAAATCGA	This study
QacGr2	*qacG*	CGAGGCCCGACCAAACT	This study
QacHf2	*qacH*	TGGCAGCTATTGCTTGGATTT	This study
QacHr2	*qacH*	TGCCAATGAACGCCCAGAAG	This study

17.5 μg ml^{-1} tetracycline and overnight shaking at 37 °C. Overnight cultures were diluted 25 times with fresh lysogeny broth supplemented with 17 μg ml^{-1} tetracycline and 0.2% arabinose. The fresh cultures were incubated in a shaking incubator with 120 r.p.m. at 37 °C. At 6 h, optical density and the activity of β-galactosidase based on the modified protocol reported recently were determined (Thibodeau *et al.*, 2004). Four biological replicates for each expression assay were carried out to produce mean values of activity expressed in Miller Units.

Sequence analysis

The sequences of cloned integron fragments were subjected to online sequence analysis, first nucleotide BLAST (basic local alignment search tool; National Center for Biotechnology Information (NCBI)) was used to check for homology with previously reported attC regions, then the NCBI ORF finder (open reading frame finder) and FGENESB (Softberry, Mount Kisco, NY, USA) were used to identify putative ORFs, which were translated and subjected to protein PSI-BLAST (position-specific iterated BLAST, NCBI).

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium). Differences in gene prevalence were tested for significance using a χ^2-test for the comparison of two proportions (from independent samples).

Results and discussion

Class 1 integron and qac gene prevalence

Class 1 integron prevalence was significantly higher in sewage sludge (1.01%) and pig slurry (0.21%) than in unamended soil (0.0036%), indicating that addition of these materials is likely to increase class 1 integron prevalence in agricultural soil (Figure 1, Supplementary Table S2). Leaching of antimicrobials from amended material into soil occurs (Blackwell *et al.*, 2007), where they continue to select for resistance. Class 1 integron prevalence was also high in QAC contaminated reed bed soil (0.65%) and prevalence of the fully functional QAC resistance gene, *qacE*, was highest at 0.44% (70% of bacteria carrying class 1 integrons). *qacE* carriage was significantly lower in sewage sludge at ~0.09% and was undetectable in pig slurry demonstrating that these genes exert a strong selective pressure for maintenance of class 1 integrons in the absence of antibiotics. The semifunctional QAC resistance gene *qacEΔ1* characteristic of class 1 integrons found in the clinic was more prevalent in pig slurry, possibly because of strong sulphonamide selection for carriage of the resistance gene *sul1* that is always carried on these integrons. *qacEΔ1* prevalence was three times that of *intI1* in pig slurry, suggesting that complex integrons with multiple copies of the gene were present or that *qacEΔ1* may also be carried by other integron classes. Heuer and Smalla (2007) established that pig slurry introduces class 1 integrons into agricultural soil, inferred by real-time PCR of *sul1*. Previous work by the authors indicated that class 1 integrons were present in several genera isolated from the pig slurry analysed in this study (Byrne-Bailey *et al.*, 2010). *qacG* was the least prevalent of the QAC resistance genes enumerated, with 0.07% and 0.22% of bacteria bearing it in reed bed sediment and sewage sludge, respectively, *qacH* illustrated an extremely high prevalence of 1.81% and 7.95% in the same samples. Both *qacG* and *qacH* were close to detection limits in pig slurry and Cotswold soil, again showing that *qac* gene prevalence is much higher in the presence of detergent and biocide residues. Previous research in sediments and biofilms from the metropolitan Sydney area found that ~50% of class 1 integrons carried a QAC resistance gene cassette, confirming that QAC selection may be the dominant selective pressure for class 1 integron carriage in wastewater and polluted surface waters (Gillings *et al.*, 2009b). The high prevalence of *qacH* seen in this study relative to *intI1* prevalence supports data by Gillings *et al.* (2009a) showing *qac* gene carriage by diverse integron classes. In addition, the possibility that *qac* genes, such as *qacH*, may be present on bacterial chromosomes or other MGEs cannot be excluded.

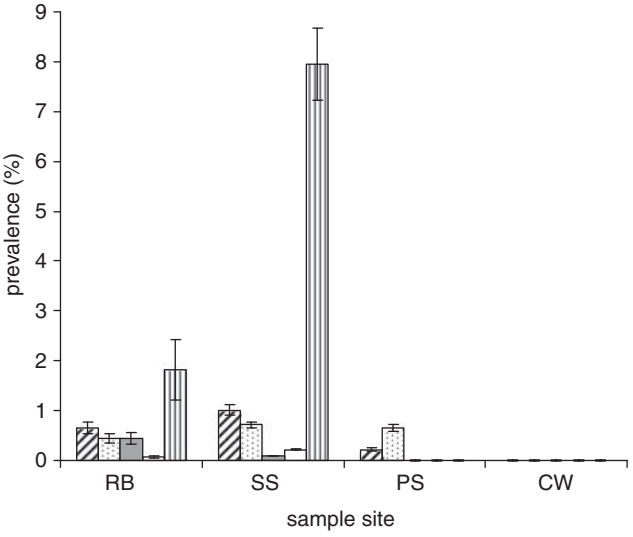

Figure 1 Gene prevalence in total bacterial population from QAC contaminated reed bed (RB), sewage sludge (SS), pig slurry (PS) and Cotswold soil (CW). Diagonal lines, *intI1*; stippled bars, *qacEΔ1*; grey bars, *qacE*; white bars, *qacG* and vertical lines *qacH*. Error bars are s.d. of four biological replicates, each composed of three technical replicates. Prevalence is statistically greater for all genes in bacteria from RB, SS and PS than from CW (χ^2-test for comparisons of two proportions; from independent samples).

Class 1 integron persistence in sludge amended soil

To investigate the impact of sewage sludge application on class 1 integron prevalence in agricultural

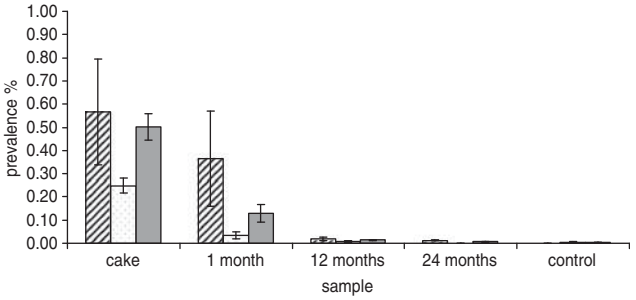

Figure 2 Gene prevalence in total bacterial population from limed, dewatered sewage sludge (cake), agricultural soil 1, 12 and 24 months after cake application. Control samples were taken from an area adjacent to the agricultural amended plots, which had not received sludge. Diagonal lines, *intI1*; stippled bars, *qacEΔ1*; grey bars *qacE*. Error bars are s.d. of three biological replicates for cake and 10 for amended soils, each composed of three technical replicates. All *intI1* prevalences are statistically different from one another.

soil, real-time PCR was conducted on 10 replicate soil cores from plots amended with limed, dewatered sewage sludge 1, 12 and 24 months previously (Figure 2, Supplementary Table S3). *IntI1* prevalence was 0.56% in limed cake used in the most recent application and 0.36% in soil cores at 1 month. Prevalence was lower in limed dewatered cake than in digested sludge collected from a wastewater treatment plant (Figure 1), but not by as much as might have been expected from liming, a method used to reduce numbers of bacterial pathogens. After 12 and 24 months *intI1* prevalence had fallen significantly to ~0.02% and 0.01%, respectively (10 times higher than in Cotswold soil), and was undetectable in a control plot on the same farm. The decrease from 12 to 24 months was small but was still significant ($\chi^2 = 1898.659$, $P < 0.0001$) and in turn the prevalence at 24 months was significantly greater than in the control plot ($\chi^2 = 5601.472$, $P < 0.0001$). The prevalence of *qacE* was higher in the limed dewatered cake than in the fully treated sewage sludge collected from four digesters, which may be because of the differences in wastewater entering the plant, or the effect of liming and dewatering impacting some microbial taxa more than others. Previous research by Ghosh *et al*. (2009) demonstrated that mesophilic digestion did not achieve reproducible decreases in *intI1* prevalence. Our study is the first to demonstrate a measurable effect of sludge application on class 1 integron prevalence in soil post application.

Promoter modification in class 1 integrons under QAC selection

A class 1 integron identified from isolates of *Pseudomonas fluorescens*, *Aeromonas hydrophila* and *Serratia proteamaculans* isolated from a QAC contaminated reed bed used to remediate textile mill effluent contained an insertion sequence (IS) between the *intI1* and *qacE* gene (Figure 3a). These strains were reported in a previous publication (Gaze *et al*., 2005). The bacterial population isolated from the reed bed was highly resistant to QACs when compared with controls. Over 95% of isolates carrying a class 1 integron carried *qacE*, which on further examination was always associated with an IS (accession no. FJ663011). Metagenomic DNA from QAC contaminated samples, that is, reed bed soil and fully treated sewage sludge, were subjected to PCR using primers targeting the class 1 integrase (*intI1*) gene and *qacE/qacEΔ1* (Rosser and Young, 1999) to investigate the possibility of discovery of further IS elements. Approximately 100 clones were sequenced, revealing 97 clones containing *intI1* adjacent to *qacE/qacEΔ1* of ~800 bp in length. Three larger clones were identified, one from reed bed soil with a 2100 bp insert containing a novel IS element inserted in between the two genes (Figure 3b; accession no: FJ663010) and two clones also from reed bed soil with 1460 bp inserts containing a gene encoding a protein related to a phage protein and a xenobiotic response element family transcriptional regulator followed by *qacE/qacEΔ1* (accession no: FJ663009). The comparative rarity of ISs in clones is in contrast to the high prevalence in isolates, however, cloning bias may account for some of this disparity or its distribution in uncultured versus cultured bacteria.

Analysis revealed that the two IS elements were ISPpu*17*, first described by Mantengoli and Rossolini (2005) as IS*1066* where it was inserted upstream of an antibiotic resistance gene in Tn*5393*d, and the second a novel IS*3* family element ISUnCu*13*. Further examination of the location of ISPpu*17* revealed that it was inserted in between the *qacE* promoter −35 and −10 hexamer forming a fusion promoter consisting of a −35 hexamer in the 3′-end of ISPpu*17* 18 bp upstream of the *qacE* −10 hexamer, 5′-**TTGCGC**TTATTCTATGAATTCA GG**CATAAT-**3′ (the bases underlined, including the −35 hexamer (in bold) were part of the right hand imperfect inverted repeat of ISPpu*17*, whereas the −10 hexamer (in bold) was part of the *qacE* gene cassette, Figure 3a). *qacE* is unusual in that it has its own weak promoter in the gene cassette, whereas transcription of most cassettes is initiated by the Pc promoter contained in the 5′ region of the integron (Guerineau *et al*., 1990). The *qacE* promoter is positioned from base 52 to 81 of the gene cassette with the *qacE* ORF starting at base 109. The Pc promoter has variable strength, with four types described (Stokes and Hall, 1989; Bunny *et al*., 1995). The Pc promoter present in the reed bed isolates and clone was the weaker of those described, −35 (TGGACA) −10 (TAAGCT) which conferred a half-maximal inhibitory concentration (IC^{50}) of 65 μg ml^{-1} against streptomycin with an *aadA2* cassette inserted downstream compared to 1000 μg ml^{-1} with the strongest promoter −35 (TTGACA) −10 (TAAACT) (Collis and Hall, 1995). ISPpu*17* belongs to the IS*30* family and is 1065 bp in

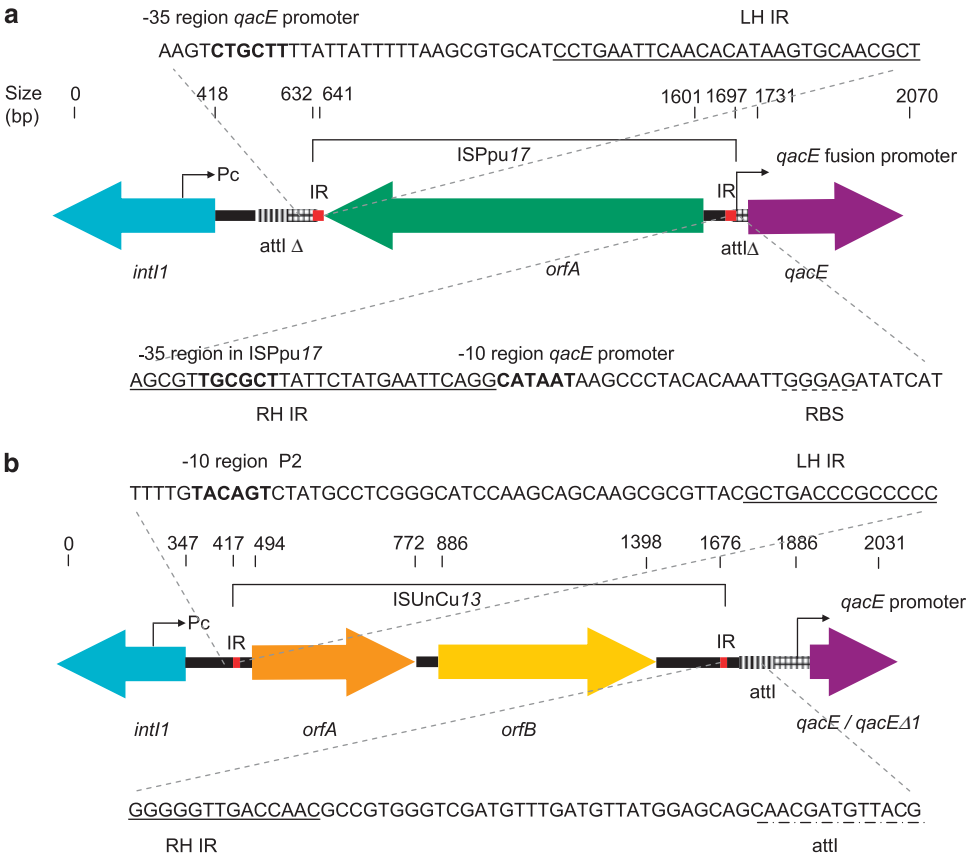

Figure 3 Location of ISPpu*17* (**a**) and ISUnCu*13* (**b**) relative to *intI1* and *qacE/qacEΔ1*. Large arrows show orientation of ORFs, inverted repeats (IR) in red and underlined in sequences; LH IR, left hand IR, RH IR, right hand IR; part of attI supplied by integron, vertical lined areas; part of attI supplied by cassette, cross hatched areas; attIΔ, attI interrupted by IS; Pc, promoter driving cassette gene expression; RBS, ribosomal-binding site, underlined by dashed line; attI, recombination site, underlined by dashed and dotted line in sequence.

size and encodes a putative 320 aa transposase. Rosser and Young (1999) also reported a novel IS*30*-like element upstream of *qacE*, *qacEΔ1* and *aadA1a*, however, little detail was given other than the fact that it was not integrated by recombination at the attI1 site as cassettes are. Mantengoli and Rossolini (2005) named their IS element IS*1066*, which was identical to that found in this study, however, van der Meer *et al.* (1991) described a different insertion sequence as IS*1066* previously. The IS*30*-like IS was also reported by Poirel *et al.* (2006) and was given the name ISPpu*17* (deposited on the IS-finder database), as it was found on a class II transposon-borne structure carrying bla_{VIM-2} in *Pseudomonas putida*. More recently, IS*1066* identical to ISPpu*17* was reported from a class 1 integron borne by *Aeromonas caviae* isolated from Australian cattle (Barlow *et al.*, 2008). In this instance, no identity of downstream genes or activity of the insertion sequence were discussed. However, these reports suggest the IS*30*-like element reported in this study may be widely disseminated in class 1 integrons.

The novel IS*3*-like element, ISUnCu*13*, from a reed-bed clone was inserted ~50 bp downstream of the P2 promoter before the attI1 integration site. The putative IS element was 1259 bp in length and contained two ORFs, with closest similarity to ISDet*3*, transposase *orfA* in *Erythrobacter litoralis* (identities = 52/92 (56%), positives = 72/92 (78%)) and putative transposase *orfB* from *E. litoralis* (identities = 83/123 (67%), positives = 95/123 (77%)).

Promoter activity of IS elements

IS elements are known to act as promoters for downstream gene expression and fusion promoters have been previously described, a study of 2,4,5-trichlorophenoxyacetic acid degradation in *Burkholderia cepacia* showed that IS*1490* inserted 110 bp before *tftA* created a fusion promoter responsible for constitutive transcription of the gene (Hubner and Hendrickson, 1997). To test for promoter activity, the IS elements described in this study were cloned into the promoter-less *lacZ* reporter vector pRW50 (Thibodeau *et al.*, 2004). Recombinant vectors were constructed containing the Pc promoter, *qacE* promoter, Pc + *qacE* promoter, 350 bp of the 3′ region of ISPpu*17* and *qacE* fusion promoter and the 3′ region of ISUnCu*13* plus the *qacE* promoter (~235 bp of ISUnCu*13*, attI1 integration site, *qacE* 59 base element and 40 bp of the *qacE* ORF). Results demonstrated that both IS elements

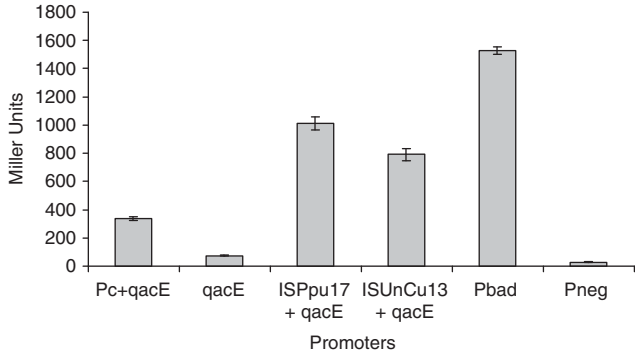

Figure 4 Promoter activity measured in *lacZ* reporter constructs; Pc, main promoter driving gene cassette expression in class 1 integrons, amplified with Pc promoter forward and Pc promoter reverse primers (Supplementary Table S1; template DNA accession no. FJ663012); *qacE*, promoter from *qacE* gene cassette, amplified with *qacE* promoter forward and reverse (accession no. FJ663012); ISPpu*17*/*qacE*, fusion promoter, amplified with ISUnCu*13* forward and *qacE* promoter reverse (accession no. FJ663011); ISUnCu*13*+*qacE*, 3′ region of ISUnCu*13* and *qacE* promoter, amplified with ISUnCu*13* forward primer and *qacE* reverse (accession no. FJ663010); pbad, promoter amplified with pBad forward and pBad reverse; neg, negative control with coding region of *araC* with no promoter amplified by *araC* forward and reverse primers. Miller Units = $1000 \times \text{absorbance}_{420}/(\text{absorbance}_{595} \times \text{reaction time} \times \text{volume})$.

dramatically increase expression of the reporter, 13.5 times in the case of ISPpu*17*and 10.5 times for ISUnCu*13* relative to the *qacE* promoter and ~3 and 2.3 times when compared with the Pc and *qacE* promoters combined (Figure 4). Overall increase in expression will be a function of the IS element promoter strength combined with the Pc and P2 promoters. This data suggests that in the presence of strong QAC selection integrons can increase the strength of cassette gene expression by maintenance of IS elements with promoter activity. The ISPpu*17* upregulates expression of *qacE* and downstream cassettes whilst ISUnCu*13* increases expression of all integrated cassettes given its location in the 5′ conserved segment, dissemination of this novel promoter arrangement to bacterial pathogens would be a worrying development.

Conclusions

We report here, for the first time, the impact of subtle differences in selective pressure for class 1 integron carriage exerted by QACs and antibiotics in the environment; and demonstrate high prevalence of class 1 integrons, even in the absence of antibiotic selection, including *qacEΔ1* clinical type class 1 integrons that carry sulphonamide resistance. Sewage sludge and animal slurries contained large numbers of class 1 integrons. Current waste disposal practices may present a significant risk to human health by recycling antibiotic resistance genes of human and animal origin to the wider environment and in particular agricultural land. A measurable difference in class 1 integron prevalence in sewage sludge amended soil relative to controls was demonstrated after a 2-year period, although numbers fell sharply after 1 month. Previous research demonstrated dissemination of putative human pathogens carrying integrons via subsurface drain flow 164 days after pig slurry analysed in this report was amended to agricultural soil (Byrne-Bailey *et al.*, 2009). Disposal of biologically active compounds, such as detergents, biocides and antibiotics combined with disposal to land of sewage sludge and animal slurries may have a significant role in the evolution and dissemination of antibiotic resistance.

It is apparent from our data that by selecting for class 1 integrons, detergents and biocides co-select for antibiotic resistance (>50% of integrons from QAC-contaminated soil carried *qacEΔ1* which is always located next to *sul1*). Once an integron is present in a bacterium, antibiotic resistance gene cassettes can subsequently be integrated and maintained in the presence of antibiotic selection. Recent research illustrated that the SOS response in bacteria, which can be induced by antibiotic exposure, can increase cassette gene excision and integration by 340 times (Guerin *et al.*, 2009), making the dissemination of integrons extremely important regardless of the antibiotic resistance genes carried. Amendment with slurry and sludge introduces large numbers of class 1 integrons and integron cassette genes into agricultural soil. On the basis of our data, each ton of sludge contains $>1 \times 10^{13}$ bacteria carrying class 1 integrons, which equates to $>1.5 \times 10^{19}$ bacteria carrying mobile genetic elements capable of conferring antibiotic resistance being added to the United Kingdom soil each year. It is well known that bacteria of agricultural origin enter water courses, the food chain and colonise farm workers (Aitken, 2003; Silvestro *et al.*, 2004; Rhoades *et al.*, 2009). It is likely that bacteria carrying class 1 integrons will enter the human population in the same way, increasing the reservoir of antibiotic resistant bacteria in the human bacterial flora.

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