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Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons?

Michael R Gillings¹, Duan Xuejun^{1,2}, Simon A Hardwick¹, Marita P Holley¹ and HW Stokes³

¹Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia; ²School of Energy and Environmental Engineering, Zhongyuan University of Technology, Zhengzhou, China and

³Department of Chemistry and Biomolecular Science, Macquarie University, Sydney, New South Wales, Australia

DNA sequencing, phylogenetic and mapping studies suggest that the class 1 integron found in pathogens arose when one member of the diverse family of environmental class 1 integrons became embedded into a Tn402 transposon. However, the timing of this event and the selective forces that first fixed the newly formed element in a bacterial lineage are still unknown. Biocides have a longer use in clinical practice than antibiotics, and a *qac* (quaternary ammonium compound) resistance gene, or remnant thereof, is a normal feature of class 1 integrons recovered from clinical isolates. Consequently, it is possible that the initial selective advantage was conferred by resistance to biocides, mediated by *qac*. Here, we show that diverse *qac* gene cassettes are a dominant feature of cassette arrays from environmental class 1 integrons, and that they occur in the absence of any antibiotic resistance gene cassettes. They are present in arrays that are dynamic, acquiring and rearranging gene cassettes within the arrays. The abundance of *qac* gene cassettes makes them a likely candidate for participation in the original insertion into Tn402, and as a source of a readily selectable phenotype. More broadly, the increasing use of *qac* and other biocides at the present time seems likely to promote the fixation of further novel genetic elements, with unpredictable and potentially adverse consequences for human health and agriculture.

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Introduction

The spread of antimicrobial resistance genes throughout human-dominated ecosystems has generated a world-wide crisis in the management of infectious disease (Davies, 2007). Plasmid, transposon and integron-borne resistance to antibiotics now occurs in the majority of Gram-negative pathogens isolated from hospitals and animal production facilities (Goldstein *et al.*, 2001; Ebner *et al.*, 2004; Essen-Zandbergen *et al.*, 2007). Understanding the origin of resistance determinants and the mobile elements that disseminate them is critical in attempting to control future recruitment and spread of resistance genes.

The class 1 integron is an important platform for recruitment of resistance genes. Class 1 integrons

encode an integrase, *IntI1*, which inserts circular gene cassettes at an integron-associated recombination site, *attI1*, and then drives their expression with an adjacent promoter, *P_c* (Stokes and Hall, 1989; Hall and Collis, 1995; Hall *et al.*, 1999). The diversity of gene cassettes present in natural environments is enormous, amounting to many thousands of different cassettes even in small areas (<50m²), each encoding novel functions (Stokes *et al.*, 2001; Michael *et al.*, 2004; Boucher *et al.*, 2007). These cassettes can be sampled by class 1 integrons (Rowe-Magnus *et al.*, 2002), whereby selection retains any that confer an advantageous phenotype, thus accounting for the accumulation of resistance cassettes by the class 1 integrons found in pathogens. The location of class 1 integrons on various mobile elements facilitates their rapid spread into new species (Liebert *et al.*, 1999; Partridge *et al.*, 2001). The ability of class 1 integrons to access diverse genes and their inherent mobility has led to their preeminent role in the spread of resistance determinants through human ecosystems. The typical class 1 integron recovered from clinical contexts is

Correspondence: MR Gillings, Department of Biological Science, Macquarie University, Sydney, New South Wales 2109 Australia. E-mail: michael.gillings@mq.edu.au

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embedded in a plasmid or transposon, and generally carries from 1 to 6 gene cassettes, primarily encoding antibiotic resistance (Recchia and Hall, 1995; Partridge *et al.*, 2001). We will refer to these as 'clinical' class 1 integrons.

Recently, class 1 integrons have been recovered from diverse environmental samples. These integrons differ from those in clinical contexts, in that they are chromosomal, and do not carry antibiotic resistance cassettes. There is good evidence to suggest that the clinical class 1 integron originated when one of these chromosomal class 1 integrons mobilized into a Tn402-like transposon (Stokes *et al.*, 2006; Gillings *et al.*, 2008a). Extant clinical class 1 integrons have a series of conserved features and sequences that strongly suggest such a single common origin. For instance, the integrase gene (*intI1*) is essentially identical in all clinical class 1 integrons, and the left hand end of all clinical class 1 integrons contains a conserved noncoding sequence that terminates in a 25-bp sequence (IRi), which is an inverted repeat of another sequence (IRt) located at the right hand end of most extant clinical class 1 integrons (Brown *et al.*, 1986, 1996, Figure 1a). These inverted repeats define the structural boundary of the clinical class 1 integron, as they are the recognition sites required for Tn402-mediated transposition (Kholodii *et al.*, 1995).

The immediate common ancestor of all clinical class 1 integrons was probably similar to a Tn402-like transposon, consisting of (in order) IRi, *intI1*, *attI1*, gene cassette(s) and their associated recombination sites (59-base elements, or *attC*), a *qacE* (quaternary ammonium compound E) cassette (which encodes resistance to biocides), a complete *tni* transposition module and IRt (Stokes *et al.*, 2006 and references therein, Figure 1a). The majority of extant class 1 integrons from clinical contexts carry various modifications to the right hand end of this

ancestral integron, including deletions in *qacE*, incorporation of a sulfonamide resistance gene (*sulI*) and partial deletions of the *tni* module (Liebert *et al.*, 1999; Partridge *et al.*, 2001; Stokes *et al.*, 2006).

Insertion of a chromosomal class 1 integron into a Tn402-like transposon followed by lateral transfer into a human commensal or pathogen provided a route into human ecosystems, but how was this rare event preserved by selection? Because *qacE* is an apparently ancestral feature of clinical class 1 integrons, we have suggested that it was the possession of a *qacE* gene cassette that provided the selective advantage for the newly generated Tn402-integron in the human environment, and that it did so by conferring resistance to biocides (Gillings *et al.*, 2008a). If this were the case, then class 1 integrons containing *qac* gene cassettes should be recoverable from environmental samples. Further, these integrons should not necessarily carry antibiotic resistance cassettes. Here, we show that diverse *qac*-encoding gene cassettes can be readily recovered using metagenomic DNA extracted from biofilms and sediments, and that *qac* cassettes are a common feature of environmental class 1 integrons.

Materials and methods

Extraction of DNA from environmental samples

Sediment and biofilm samples ($n=51$) were all obtained from a range of sites in the metropolitan area of Sydney, NSW, Australia. Samples included stream sediments from Cowan Creek, North Turramurra (RA: Hardwick *et al.*, 2008), biofilms from Mars Creek, North Ryde (BF: Gillings *et al.*, 2008b), contaminated soil from Balmain (BAL: Yeates and Gillings, 1998), biofilms from a groundwater treatment plant in Botany (OR: Gillings *et al.*, 2006) and a

Figure 1 Schematic representation of class 1 integrons and their gene cassettes, showing the locations of primers used to amplify integron constituents. (a) Transposon Tn402 that includes a class 1 integron. This element carries the terminal inverted repeats (IRi and IRt), a class 1 integron integrase gene (*intI1*), a primary recombination site (*attI1*; small diamond), a cassette array (usually encoding antibiotic resistance) with each cassette bounded by secondary recombination sites (*attC*; circles), a cassette for quaternary ammonium compound resistance (*qacE*) and a transposition module (*tni*). (b) An environmental class 1 integron embedded into a bacterial chromosome carries the integrase gene, flanking regions and a cassette array that does not usually encode antibiotic resistance. The primer combination MRG284/285 amplifies complete arrays from both these kinds of class 1 integrons. Dotted vertical lines denote the extent of sequence homology between (a) and (b), and define the boundaries of the class 1 integron (Gillings *et al.*, 2008a, b). (c) A representation of a *qac* gene cassette. Primers in the 287–295 series amplify coding regions of different *qac* groups, and *qac* genes associated with gene cassettes. Differences in the product sizes allow identification of each group (see Table 1, Figure 3).

biofilm from a suburban pond in Seven Hills. None of the sampling sites was downstream from hospitals, sewage treatment works or animal production facilities. Samples were stored at 4 °C before extraction of DNA, normally within 24 h. DNA was extracted from 400 mg subsamples using bead beating in a FastPrep machine (Bio 101, Morgan Irvine, CA, USA) (Yeates and Gillings 1998). DNA yield was assessed using agarose electrophoresis.

PCR amplifications from environmental DNA and pure cultures

An internal fragment of the class 1 integron-integrase gene, *intI1*, was amplified using primers HS463a and HS464 as described (Table 1, Stokes *et al.*, 2006). Cassette arrays associated with class 1 integrons were amplified using primers MRG284 and MRG285 (Figure 1 and Table 1). PCRs were performed in 50 µl reactions with *GoTaq* DNA polymerase (Promega, Madison, WI, USA) in the buffer supplied with the enzyme, 25 pmol of each primer and a final Mg²⁺ concentration of 1.5 mM. PCRs were carried out using an Omn-E thermocycler (Hybaid, Middlesex, UK) with the following cycling programs: 94 °C for 3 min (one cycle); 94 °C for 30 s, 60 or 65 °C for 30 s, 72 °C for 90 s (35 cycles); 72 °C for 5 min (one cycle). Positive controls were used in every amplification. These included a typical clinical class 1 cassette array located on plasmid R388 (accession no. BR000038) and a representative environmental cassette array from the chromosomal class 1 integron in *Thauera* sp B4 (accession no. EU327991) (Gillings *et al.*, 2008a).

Genes in the *qac* family were amplified using primers MRG287/MRG288 (*qacG/E2* homologs, positive control BF12_C8, accession no. FJ172380) or MRG289/MRG290 (*qacH* homologs, positive control BF12_C5, accession no. FJ172378) or MRG291/292

(*qacE1* homologs, positive control BF13_C3, accession no. FJ172384; see Table 1). PCR conditions were as above except that all six *qac* primers were used in a multiplex reaction containing 10 pmol of each primer and an annealing temperature of 55 °C was used. To confirm that the targeted *qac* genes resided in gene cassettes, the forward primers used above were paired with *attC*-specific reverse primers, using the same conditions and positive controls (Table 1). Small subunit ribosomal RNA genes (16S rDNA) were amplified using primers f27 and r1492 (Lane, 1991). The efficiency of PCR was assessed by electrophoresis on 2 or 2.5% agarose gels.

Real-time quantitative PCR

Real-time quantitative PCR assays were targeted at 16S rDNA, *intI1* and *qac* gene cassettes from biofilm samples, following the methods given in Hardwick *et al.* (2008). *Pseudomonas stutzeri* Q (Holmes *et al.*, 2003) was used as a positive control for 16S rDNA, plasmid R388 was used as a positive control for *intI1* and plasmid BF12-5 was used as a positive control for *qac* gene cassettes. Standards of known copy number were prepared and run in triplicate to generate standard curves. Assays were performed in an ABI7700 Prism sequence detector (Applied Biosystems, Foster City, CA, USA). Each environmental DNA sample was tested in triplicate using 25 ng of DNA template. Programming, detection and analysis were performed as described previously (Hardwick *et al.*, 2008).

Cloning of PCR products

PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega) and ligated into T-tailed TOPO plasmid vectors (Invitrogen, Carlsbad, CA, USA). Transformation into

Table 1 Details of PCR primer combinations

Primers	Sequence (5'–3')	Target	Size (bp)
HS463a	F CTGGATTTCGATCACGGCACG	<i>intI1</i>	473
HS464	R ACATGCCGTGTAAATCATCGTCG		
MRG284	F GTTACGCCGTGGGTTCGATG	Class 1 integron	Variable ^a
MRG285	R CCAGAGCAGCCGTAGAGC	Cassette array	
MRG284	F GTTACGCCGTGGGTTCGATG	Class 1 integron	Variable
MRG286	R CCACTGCCGCCATTGGGC	cassette array	
MRG287	F TTGGTTATTTCTGGCTACG	<i>qacG</i> family	281
MRG288	R CGCTGATAATGAAGCCGAC		
MRG289	F AAGTCCAGCCATGGATTC	<i>qacH</i> family	235
MRG290	R GGCGACGCCACTGACG		
MRG291	F AGCTTGCCCCTTCCGC	<i>qacE</i> family	193
MRG292	R AGCCCCATACCTACAAAG		
MRG287	F TTGGTTATTTCTGGCTACG	<i>qacG</i> cassettes	354
MRG293	R TCGACGGAATGGTTAGATG		
MRG289	F AAGTCCAGCCATGGATTC	<i>qacH</i> cassettes	301
MRG294	R GCTTGAATGAATTGTTAGATG		
MRG291	F AGCTTGCCCCTTCCGC	<i>qacE</i> cassettes	277
MRG295	R TGCAACGACTTGTAGATG		

^aProduct sizes vary according to number and type of gene cassettes in the amplified array.

chemically competent *Escherichia coli* OneShot TOP10 cells (Invitrogen) was carried out in accordance with the manufacturer's instructions. Twenty-four random clones were chosen from each environment and screened for the presence of inserts using PCR with the original primers on boiled colony lysates. These were prepared by suspending one colony in 200 µl of sterile water, incubation at 99 °C for 10 min, snap chilling on ice and centrifugation for 5 min at 12 000 *g*. A 1-µl aliquot of the supernatant was used as a template for PCR.

DNA sequencing of selected clones

Plasmids were purified from 5 ml overnight liquid cultures using the Wizard Plus Minipreps DNA Purification System (Promega) as per the manufacturer's instructions. Sequencing reactions contained ~200 ng of plasmid DNA and 4 pmol of pcrfnew forward or pcrR30 reverse vector primers (Gillings *et al.*, 2005). Sequencing was performed on an ABI 3130xl Genetic Analyzer using BigDye v 3.1 chemistry (Applied Biosystems). Sequencing reactions, unincorporated dye-terminator removal and capillary electrophoresis were carried out at the Macquarie University DNA Analysis Facility. Sequences were interrogated by searching against nonredundant nucleotide or protein databases using blastn and blastx algorithms (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic and molecular evolutionary analyses were conducted using the bioinformatics tools available through Biomanager, on the ANGIS web site (<http://www.angis.org.au/>).

Data deposition

DNA sequences reported in this study were deposited with GenBank as accession numbers FJ172371-FJ172422.

Results

Primers were designed to specifically amplify class 1 integron gene cassette arrays. Primer sequences were based on an alignment of the termini of known chromosomal class 1 cassette arrays and arrays carried on plasmids or transposons from clinical contexts (Gillings *et al.*, 2008a). These primers amplify arrays from chromosomal integrons and Tn402-like elements, by targeting a region in *attI1* proximal to the array (MRG284), and a region beyond the final *attC* site in the array (MRG285) (Figure 1). PCR assays using these primers produced complex amplification patterns from metagenomic DNA, generating multiple bands between 400 and 2000 bp from individual samples, suggesting that multiple class 1 gene cassette arrays were present. All 51 sediment and biofilm samples tested generated products in the assay (data not shown). PCR products from selected amplifications were cloned and DNA sequenced to determine the identity of cassettes within the arrays.

Fifty-two cassette array clones from a total of 18 independent environmental samples were sequenced. The arrays contained 1–3 cassettes bounded by characteristic *attC* sites, but in other respects were not typical of class 1 cassette arrays from clinical isolates. Two of the 70 cassettes detected encoded potential antibiotic resistance determinants. Both of these were *aadA10* cassettes with greater than 99.9% nucleotide identity to resistance cassettes commonly found in pathogens, suggesting they originated from a human or animal source. Two further cassettes, each from independent samples, encoded putative cupin barrel proteins (Dunwell *et al.*, 2001). Sequence homology placed them in COG1917, Cupin 2 barrel proteins, a family that has never previously been associated with integrons. The function of this family is currently unknown. Thirty-nine of the cassettes encoded conserved hypothetical proteins, or contained open reading frames for which no homology could be found in sequence databases. Such cassettes are typical of chromosomal integrons from environmental samples. The final 27 cassettes all encoded efflux pumps of the *qac* family, such that in total, about 50% of the class 1 arrays from environmental samples carried a *qac* cassette (Supplementary Table 1).

Comparison of the cassette arrays showed that the same cassette (>99% nucleotide sequence homology) could be found in a number of different contexts and located adjacent to a number of different cassettes. The same *qacG/E2* cassette could be found as the sole cassette within an array, or adjacent to one of the three different cassettes (Figure 2). Similarly, cassettes encoding *qacH*, cupin barrel proteins and hypothetical proteins could also be found in a number of different contexts (Figure 2). Further examples of this phenomenon (Supplementary Table 1) suggested that there is a dynamic process of cassette acquisition and rearrangement in these arrays.

Phylogenetic analysis of the *qac* cassettes revealed three clades, corresponding to groups commonly annotated as *qacE1*, *qacG/E2* and *qacH*, respectively. Alignments between these cassettes were used to design primers to specifically amplify the coding regions of each group, and to distinguish between the various groups based on amplicon size (Table 1 and Figure 1). To assess the distribution and abundance of each *qac* group in the natural environment, these primers were used in a multiplex PCR on all biofilm and stream sediment DNA samples (*n*=48). Bands of the expected size(s) amplified from the majority of samples, although the strength of amplification varied. In addition, the relative proportions of the three different *qac* groups were different in different samples. For instance, some samples generated three bands of equal strength, suggesting similar concentrations of the three *qac* groups, whereas other samples were dominated by one of the *qac* groups (Figure 3).

Figure 2 Schematic maps of class 1 integron cassette arrays from environmental samples. Maps were constructed on the basis of sequence data for cassette arrays amplified from environmental samples using primers MRG284/285 (Table 1 and Supplementary Table 1). Symbols are as follows: black diamonds, *attI1*; gray circles, *attC* (59-bp); block arrows are open reading frames within cassettes, labeled with gene names. All *qac* genes are stippled; gray shading between cassettes denotes greater than 99% nucleotide sequence homology. The two different sets of *qacH* cassettes (i and ii) in the bottom part of the figure exhibit 96% nucleotide sequence homology. Individual *qac* gene cassettes can be found in a variety of different contexts, suggesting a dynamic process of cassette acquisition and rearrangement in these arrays.

Figure 3 Representative PCR amplifications from metagenomic DNA samples. A series of environmental DNA samples were used as a template for amplification of 16S rDNA and the integron-integrase gene, *intI1*. Multiplex PCR was used to amplify coding regions of the *qac* E1, G/E2 and H families, and to amplify these same regions from within gene cassettes (*qac*^{cass}). Tracks are from the following environmental samples: (A) BF7, (B) BF2, (C) BF4, (D) BF11, (E) BF12, (F) BF16, (G) BF13, (H) BF17, (I) BF15 and (J) BF24. Tracks (K–M) are positive controls as detailed in Materials and methods section, (m) = 100 bp molecular weight ladder. Sizes are given at the left hand side.

The overall intensity of response generated in *qac* PCR correlated extremely well with the response generated by amplification of the same samples with *intI1* primers (Figure 3). However, this variation was not correlated with the intensity of 16S rDNA amplifications (for example, tracks B vs C in Figure 3). This suggested that there was variation in the abundance of class 1 integrons between samples, but that the proportion of class 1 integrons that contained *qac* cassettes did not change. To confirm that the *qac* PCR products did represent *qac* coding regions embedded within gene cassettes, a second multiplex PCR was performed using the forward *qac* primers and a set of three reverse primers anchored in the *attC* sites specifically associated with each *qac* group (Figure 1). The intensities of bands generated in this assay matched those of the *intI1* and *qac* assays (Figure 3). The best explanation for the PCR results, in conjunction with the sequencing results, is that a large and near constant proportion of class 1 integrons from environmental samples carried *qac* gene cassettes.

To further investigate the abundance and diversity of class 1 cassette arrays and their associated *qac* gene cassettes, we performed real-time quantitative PCR directed at *intI1* and *qac* gene cassettes in all the biofilm samples ($n=24$). Numbers of cells containing *intI1* were normalized with respect to 16S rDNA quantitative PCR performed on the same samples (Hardwick *et al.*, 2008). On the basis of this analysis, between 1 and 9% of cells in the biofilms were *intI1* positive (mean $\pm$ s.e. = $4.5 \pm 0.55\%$). The proportion of cells in the various biofilms that carried *qac* gene cassettes was approximately half of this value (mean $\pm$ s.e. = $2.2 \pm 0.59\%$). These data agree with those from the sequencing analysis, suggesting that about 50% of *intI1*-positive cells from environmental samples carry a *qac* gene cassette.

Discussion

It is broadly accepted that resistance to antimicrobial agents is one of the most important medical issues facing humankind (Davies, 2007). Lateral gene transfer has played a critical role in the dissemination of these resistance determinants, but we still have little information about the sources of resistance genes and their mobile vectors. Examination of enterobacterial isolates collected during the 1920s found no antibiotic resistance genes (Datta and Hughes, 1983; Hughes and Datta, 1983); so clearly, over the last 90 years such genes have been repeatedly sampled from the vast reservoir of microbial diversity that resides in natural environments. Studying the pool of potential resistance genes and mobile vectors could help predict future threats posed by lateral gene transfer events (D'Costa *et al.*, 2006).

Previous examination of the diversity of *intI1* in environmental samples showed that the ancestor of the class 1 integrons now circulating in human commensals and pathogens was probably an integron residing on the chromosome of a β -proteobacterial species. Under this model, the chromosomal class 1 integron was inserted into a Tn402 transposon, which then found its way into a human commensal or pathogen (Stokes *et al.*, 2006; Gillings *et al.*, 2008a). It was suggested that the possession of a *qac* gene cassette provided a selective advantage for this novel DNA element (Gillings *et al.*, 2008a) by conferring resistance to the *qac* used as disinfectants in hospitals from the 1930s onwards (Russell, 2002). We now have strong supporting evidence for this hypothesis.

Diverse *qac* gene cassettes that are resident in class 1 integron cassette arrays can be readily recovered from environmental samples. These *qac* cassettes were generally not associated with any known antibiotic resistance cassettes, as might be expected if the arrays we detected were being shed from hospitals, animal production facilities or sewage systems. About half of all class 1 integron arrays from environmental samples carry *qac* cassettes, such that they are the most common cassette type recovered. Further, individual *qac* cassettes occur in many different contexts, suggesting a dynamic process of cassette acquisition and rearrangement in these arrays. Consequently, insertion of any individual environmental class 1 integron into Tn402 was more likely than not to carry a *qac* gene cassette in its array. The widespread distribution of diverse *qac* cassettes hints at a general role for these efflux pumps in environmental bacteria. It is thought that efflux pumps defend against toxins in natural ecosystems (Gilbert and McBain, 2003), but the natural targets of *qac* efflux systems are currently unknown.

The rise in resistance to antibiotics is paralleled by a similar trend in resistance to biocides (Higgins *et al.*, 2001; Bjorland *et al.*, 2005), prompting concerns that the increasingly widespread use of biocides enhances the selection and dissemination of antibiotic resistant bacteria (Russell, 2002; Gilbert and McBain, 2003). There are two widely discussed mechanisms by which this coselection might occur. The first of these is the acquisition of generic resistance mechanisms that function to exclude or remove both antibiotics and biocides, such as changes to membrane properties or the acquisition of efflux pumps (Russell *et al.*, 1998; Langsrud *et al.*, 2004; Poole, 2005). The second mechanism involves coselection of antibiotic resistance genes through their linkage with biocide resistance determinants on the same plasmid or transposon. The best examples of such a case are class 1 integrons from clinical contexts, where genes for resistance to *qac*, or fragments thereof, are often closely linked to gene cassettes encoding antibiotic resistance (White and McDermott, 2001; Gaze *et al.*, 2005).

There is, however, a third mechanism whereby the human use of biocides may promote dissemination of mobile genetic elements and hence resistance genes, and this is by promoting the selection of novel lateral gene transfer events from environmental bacteria. All newly acquired mobile elements and resistance genes ultimately originate from natural environments. Their passage from natural environment either directly into a pathogen or through a human commensal is reliant on (i) an initial, low frequency, transfer event and (ii) selection for any newly acquired phenotype. Consequently, the more selection we apply, the more diverse lateral transfer events become fixed in human ecosystems. These transferred elements can then participate in the rearrangements and further exchanges that typify the mosaic structure of mobile DNAs (Toussaint and Merlin, 2002), multiplying the evolutionary opportunities for interactions between mobile elements, transposases, site-specific recombinases and any associated genes that confer novel phenotypes (Frost *et al.*, 2005; Tetu and Holmes, 2008). Use of biocides may have driven the fixation and spread of the class 1 integron, now responsible for a major part of antibiotic resistance. Current trends for broader and more indiscriminate use of biocides in homes, businesses and hospitals may drive the emergence of new genetic elements, with unpredictable consequences for human welfare.

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