Class 1 Integrons in Environments with Different Degrees of Urbanization

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Abstract

Background: Class 1 integrons are one of the most successful elements in the acquisition, expression and spread of antimicrobial resistance genes (ARG) among clinical isolates. Little is known about the gene flow of the components of the genetic platforms of class 1 integrons within and between bacterial communities. Thus it is important to better understand the interactions among “environmental” intI1, its genetic platforms and its distribution with human activities.

Methodology/Principal Findings: An evaluation of two types of genetic determinants, ARG (sul1 and qacE1/qacED1 genes) and lateral genetic elements (LGE) (intI1, ISCR1 and tniC genes) in a model of a culture-based method without antibiotic selection was conducted in a gradient of anthropogenic disturbances in a Patagonian island recognized as being one of the last regions containing wild areas. The intI1, ISCR1 genes and intI1 pseudogenes that were found widespread throughout natural communities were not associated with urbanization (p > 0.05). Each ARG that is embedded in the most common genetic platform of clinical class 1 integrons, showed different ecological and molecular behaviours in environmental samples. While the sul1 gene frequency was associated with urbanization, the qacE1/qacED1 gene showed an adaptive role to several habitats.

Conclusions/Significance: The high frequency of intI1 pseudogenes suggests that, although intI1 has a deleterious impact within several genomes, it can easily be disseminated among natural bacterial communities. The widespread occurrence of ISCR1 and intI1 throughout Patagonian sites with different degree of urbanization, and within different taxa, could be one of the causes of the increasing frequency of multidrug-resistant isolates that have characterized Argentina for decades. The flow of ARG and LGE between natural and clinical communities cannot be explained with a single general process but is a direct consequence of the interaction of multiple factors operating at molecular, ecological, phylogenetic and historical levels.

Introduction

In addition to the global causes of death by viruses, bacteria and parasites, which are a huge burden on public health, the progressive increase of multidrug resistance in all geographical regions has been identified as a public health priority according to the World Health Organization, 2011 (http://www.who.int/drugresistance). In recent years, research on the function of antibiotic resistance in non-clinical environments has begun to receive attention [1,2,3,4]. This interest is based on the idea that a better understanding of the diversity of patterns and biological functions of antibiotic resistance mechanisms may eventually help to control its threats towards human and also animal health. The role of the environment as a reservoir of strains that have never before been isolated from humans was demonstrated during the outbreak caused by enterotoaggregative Escherichia coli that had acquired the genes to produce Shiga toxins in Germany in May 2011. This episode also stresses the negative consequence of having mechanisms of antimicrobial resistance in these isolates (blaTEM-1 and blaCTX-M-15), which most likely helped the bacteria to survive and persist in different habitats [5].

Most of the new research into natural bacterial communities has focused on antimicrobial resistance genes (ARG) that confer resistance to antimicrobial drugs, mainly associated with protection against natural antibiotics or with functional properties among the metabolic pathways of environmental bacteria [2,6,7]. In contrast, scarce research has focused on the natural occurrence and role of the genetic platforms (transposons, integrons) that participate in the capture and dispersion of these genes within and among genomes, usually known as mechanisms of lateral genetic transfer [8]. To our knowledge, there are no prospective studies that analyses the independent occurrence of genetic components of a particular genetic platform, i.e. the lateral genetic transfer...
determinants such as integrons or transposons with the ARG elements, considering both ecological and molecular parameters.

Lateral genetic transference is a widespread phenomenon that is not only largely responsible for the ability of pathogenic and opportunistic bacteria to resist clinical antibiotic pressures [9], but also enables exchange of the accessory genome, which is a major contributor to bacterial evolution [10]. Of the different mechanisms involved in lateral genetic transfer, the class 1 integrons are one of the most successful elements in the acquisition, abundance, maintenance and spread of antimicrobial resistance gene cassettes among gram-negative bacilli isolated from clinical samples [11,12,13,14]. Although their role has not been yet investigated, class 1 integrons have been also found in gram-positive clinical strains, including methicillin-resistant Staphylococcus aureus and Corynebacterium species [15,16,17,18,19,20], from several hospitals around the world. The basic structure of an integron possesses a gene for an integrase (intI1), a recombination site (attI) and a promoter (Pc) that permits the expression of gene cassettes incorporated in the variable region [21]. Several genetic structures have been described at the 3’ end of the variable region of class 1 integrons [22,23,24,25,26,27,28]. There are three genetic platforms containing and spreading the class 1 integrons described in clinical samples from Argentina (Figure 1): (i) the most common one exhibits the well-known 3’-conserved segment (3’-CS) at the end of the variable region, which contains the qacEΔ1 gene that is a deleted form of the quaternary ammonium compounds resistance gene cassette, qacE, followed by the sul1 gene that confers resistance to sulphonamides, and finally the oprF3 of unknown function [12] (Figure 1A); (ii) the complete or incomplete module of Tn402, intC-intM-intD-intA [29] (Figure 1B); and (iii) the 3’-CS can be invaded by the putative site-specific recombinase ISCR1, which adds a second variable region that can contain an important variety of antimicrobial resistance genes such as blqCRX-M-2 and qacB10, which are known as unusual or complex class 1 integrons [30,31,32,33] (Figure 1C). Neither class 1 integrons nor unusual class 1 integrons allow intracellular mobilization of the intI1 gene per se. However, almost all clinical members of class 1 integrons harbour the IR of the Tn402 transposon, transforming this genetic platform into a mobile element when the tns genes are provided in trans [25,34]. In addition, Tn402 targets plasmid and transposon resolution sites (res) [35], expanding the range of lateral gene transfer between clinical and natural communities [1]. It is very important from a clinical standpoint that the association of genes in the same genetic platform is co-selected under antibiotic pressure.

The relevant role of natural communities as a reservoir and original source of class 1 integrons was recently identified [1,15,36,37]. Since then, their distribution has been reported in environments with different degrees of human disturbance [1,15,36,37,38,39,40,41,42]. Overall, it is assumed that 2.65% of eubacterial cells in non-clinical samples contain a class 1 integron [41]. However, factors involved in the distribution of non-clinical class 1 integrons within natural communities remain largely unknown. What is known with certainty is that the class 1 integrons confer a benefit to the host cell due to their ability to acquire gene cassettes that could provide advantages for survival in hostile environments [43,44,45,46,47].

Concerning the molecular evolution of these elements, class 1 integrons were found to be chromosomally located, pre-dating the association with the Tn402-like transposon in non-clinical samples, suggesting that the ancestor of the clinical class 1 integron was more like a typical chromosomal integron [1]. The understanding of the molecular and environmental properties that contribute to the global success of class 1 integrons is the first step towards compiling a comprehensive story of how genes, genetic platforms, bacterial populations and selection pressures interact with human activities. However, it is difficult to assess the directionality of the flow of genes among natural environment and human habitats. The different alleles of the intI1 gene from natural communities led to the identification of the sources of both “environmental” and “clinical” class 1 integrons [48].

The aim of this study was to analyse the relationships among “environmental” intI1, its genetic platforms and its distribution with human activities in areas with different levels of urbanization (Table 1). Our methodology was based on two strategies: (i) we worked at two scales of analyses, molecular and ecological levels, and (ii) we evaluated two types of genetic elements from the same samples, ARG (sul1 and qacE1 genes) and genetic elements associated to lateral genetic transfer such as intI1, ISCR1 and tniC genes, called lateral genetic elements (LGE) for convenience in this paper and which comprise the genetic platforms of class 1 integrons. The first strategy allows the simultaneous analyses of ecological patterns and molecular mechanisms. The second strategy is based on the expectation that ARG will respond differently to LGE regarding the geographical variation of urbanization due to the different roles of these elements. We hypothesized that, if LGE serve as a general response mechanism to environmental stress, they should be present in both “clean” and urbanized habitats, as far as bacteria meet stressful conditions in “clean” habitats (for example, extreme seasonal or daily variations in weather conditions). Thus, they should present a weak relationship with the degree of urbanization. In contrast, ARG should be closely related to antibiotic pressure, and thus will present a strong link to geographical variations in urbanization and human presence. The field work was conducted in Tierra del Fuego, a Patagonian island from Argentina and Chile (Figure 2), which is recognized as being one of the last places on Earth that contains land areas that can still be considered wild or “clean”, given its great extensions of intact, natural vegetation and large vertebrate assemblages, along with a low human population density [49].

Results and Discussion

Taxonomic Distribution of the Components of the Genetic Platforms of Class 1 Integrons

We identified the following bacterial taxa using culture-dependent methods: γ and β classes within the Proteobacteria phylum (34 and 11 isolates, respectively), the Flavobacteria class within the Bacteroidetes phylum (3 isolates), Arthrobacter, Streptomyces, Microbacterium and Micrococcus genera within the Actinobacteria phylum (9 isolates) and the Pseudomonas genus within the Firmicutes phylum (1 isolate). Whole intI1 genes were identified in 11 isolates of γ-proteobacteria (Table 2). Although intI1 genes were more abundant in Pseudomonas than among other families of the γ-proteobacteria (6/11), Enterobacteriaceae intI1 alleles showed high sequence diversity (Table 2). The intI1 pseudogenes were mostly found in γ-proteobacteria, but they were also identified in two β-proteobacteria isolates and in one Actinobacteria isolate, evidencing the widespread dissemination of this genetic element. The remaining genetic determinants showed different patterns of taxonomic distribution as ISCR1 and sul1 were found in γ-proteobacteria and in Actinobacteria, and tniC in γ-proteobacteria and Flavobacteria (Table 2): The qacE1/qacEΔ1 gene showed different frequencies between taxa (Table 3). Its frequency in γ-proteobacteria (18/74) was almost half the frequency in the other taxa (10/23).
A previous study from Australia found a prevalence of intI1 genes in non-clinical isolates of β-proteobacteria strains in a similar culture-based method [1]. They also found a great dispersion of qac genes in environmental samples, and proposed that selection for qac resistance before the antibiotic era contributed to the mobilization and widespread of class 1 integrons among the environmental Proteobacteria. They argued that when antibiotics began to be administrated it would be almost inevitable that class 1 integrons would come to play a major role in the dissemination of antibiotic resistance [50].

With the information available from ours and other studies [48,50], it is possible to build a hypothesis on the role of environmental γ and β-proteobacteria as sources of clinical intI1 genes. In addition, we found that at each sampling site positive for intI1, ISCR1 was also detected and at least one sul1 and/or qacE1/qacED1 gene was identified. Although the succession of molecular steps involved in the acquisition of components of the genetic platforms of class 1 integrons circulating in hospitals nowadays (Figure 1) could not be determined, our study showed a scheme in which the c and b proteobacteria harbouring intI1 genes share habitats with several other genera belonging to c, b-proteobacteria, Actinobacteria and Bacteroidetes, which in turn have the ISCR1, sul1, tniC and/or qacE1/qacED1 genes. These genes could have been co-acquired in one bacterial cell by mechanisms associated with lateral genetic transference and later selected by antimicrobial pressure within clinical settings and/or by human activities.

The Sul1 Gene is the Only Genetic Marker Associated with Urbanization

The frequency of occurrence of sul1 was significantly and positively related to the level of urbanization, whereas the other genes, intI1, ISCR1, qacE1/qacED1 and tniC, were not significantly related to this variable (p>0.05) (Figure 3; Table 3). All 10 sul1
genes obtained by PCR were sequenced and 7 of them exhibited more than two mutations in 581-bp length compared to the sul1 from clinical isolates (accession number JF262166). In addition, high sequence diversity of the sul1 gene (83% identity compared to the sul1 sequence in accession number JF262166) was found in one strain isolated from a site with low-level anthropogenic disturbances (3SC2 isolate, Table 2). In order to analyse the relationship between “environmental” and “clinical” types of genes we performed a phylogenetic analysis with sul1 alleles from the Genbank and from our work (Figure 4). More than 30 alleles of the sul1 gene were identified in this analysis. Only the sul1 allele from the 3’-CS of the integrons was found in both clinical and non-clinical isolates.

The high frequency of the sul1 genes in urban sites could be the consequence of gene flow of the “clinical” sul1 allele from the hospital towards the open environment to which are added the

Table 1. Sampling sites of Tierra del Fuego Island.

| Site Name          | Date       | Geographic location | Houses | Roads | Other buildings | Urbanization |
|--------------------|------------|---------------------|--------|-------|-----------------|--------------|
| 1 Ushuaia 1 (Pipo River) | 22/01/06   | S54’ 49’ 58’ W68’ 21’ 05’ | >100   | >10   | 10              | High         |
| 2 Road N’ 26 1 (Turbio River) | 25/01/06 | S54’ 47’ 37’ W67’ 15’ 36’ | 0      | 1     | 0               | Low          |
| 3 Road N’ 26 2 (Turbina Maicasen) | 25/01/06 | S54’ 35’ 33’ W67’ 03’ 38’ | 6      | 1     | 2               | Medium       |
| 4 Escondido Lake 1 (hotel) | 27/01/06   | S54’ 40’ 49’ W67’ 48’ 48’ | 15     | 2     | 2               | Medium       |
| 5 Escondido Lake 2 (stream) | 27/01/06 | S54’ 41’ 2’ W67’ 49’ 39’ | 0      | 2     | 0               | Low          |
| 6 National Park 1 (Ovando River) | 01/02/06 | S54’ 50’ 36’ W68’ 34’ 42’ | 0      | 2     | 0               | Low          |
| 7 National Park 2 (Ensina Bay stream) | 01/02/06 | S54’ 50’ 48’ W68’ 28’ 56’ | 0      | 1     | 1               | Low          |
| 8 National Park 3 (Pipo River) | 02/02/06 | S54’ 49’ 2’ W68’ 28’ 37’ | 0      | 1     | 0               | Low          |
| 9 Baliza Davidson stream (Moat) | 04/02/06 | S54’ 56’ 24’ W66’ 54’ 30’ | 0      | 1     | 0               | Low          |
| 10 Ushuaia 2 (Ushuaia River) | 20/01/06 | S54’ 47’ 37’ W68’ 15’ 36’ | >100   | >10   | 10              | High         |

Degree of urbanization was quantitatively estimated by counting the number of buildings and roads that were contained within a 1 km circular area around each site (see Material and Methods).

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| Isolate | Genus       | Taxonomic Class | intI1 | Mutations* | intI1 pseudogene | qacE/ qacEΔ1 | sul1 | ISCR1 | tniC |
|---------|-------------|-----------------|-------|------------|------------------|--------------|------|-------|------|
| 1AC1    | Arthrobacter| Actinobacteria   | 0     |            |                  |              |      |       |      |
| 1AC2    | Aeromonas   | γ- proteobacteria| 1     | 0          |                  |              |      |       |      |
| 1AC3    | Vibrio      | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 1AC4    | Vibrio      | γ- proteobacteria| 1     | 1          |                  |              |      |       |      |
| 1AC5    | Aeromonas   | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 1AL1    | Enterobacter| γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 1AL2    | Aeromonas   | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 1AL3    | Aeromonas   | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 1AL4    | Streptomyces| Actinobacteria   | 0     | 1          |                  |              |      |       |      |
| 1AL5    | Microbacterium| Actinobacteria       | 0      | 1          |                  |              |      |       |      |
| 1ALEv1  | Arthrobacter| Actinobacteria   | 0     |            |                  |              |      |       |      |
| 1SC1    | Arthrobacter| Actinobacteria   | 0     |            |                  |              |      |       |      |
| 1SC2    | Arthrobacter| Actinobacteria   | 0     |            |                  |              |      |       |      |
| 1SC3    | Arthrobacter| Actinobacteria   | 0     | 1          |                  |              |      |       |      |
| 1SL1    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 1SL2    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 1SL3    | Arthrobacter| Actinobacteria   | 0     | 1          |                  |              |      |       |      |
| 1SL4    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 1SL5    | Pseudomonas | γ- proteobacteria| 1     | 0          |                  |              |      |       |      |
| 1SLev1  | Micrococcus | Actinobacteria   | 0     | 1          |                  |              |      |       |      |
| 2AC1    | Janthinobacterium| β- proteobacteria | 0      | 1          |                  |              |      |       |      |
| 2AC2    | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 2AC3    | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 2AL1    | Janthinobacterium| β- proteobacteria | 0      | 1          |                  |              |      |       |      |
| 2AL2    | Janthinobacterium| β- proteobacteria | 0      | 1          |                  |              |      |       |      |
| 2AL3    | Yersinia    | γ- proteobacteria| 1     | 1          |                  |              |      |       |      |
| 2AL4    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 2AL5    | Janthinobacterium| β- proteobacteria | 0      | 1          |                  |              |      |       |      |
| 2ALEv1  | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 2AN1    | Flavobacterium| Flavobacteria     | 0      | 1          |                  |              |      |       |      |
| 2AN2    | Flavobacterium| Flavobacteria     | 0      | 0          |                  |              |      |       |      |
| 2AN3    | Paenibacillus| Bacilli          | 0      |            |                  |              |      |       |      |
| 3AL1    | Janthinobacterium| β- proteobacteria | 0      | 1          |                  |              |      |       |      |
| 3AL2    | Serratia    | γ- proteobacteria| 0     | 0          |                  |              |      |       |      |
| 3AL3    | Yersinia    | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 3AL4    | Burkholderia| β- proteobacteria| 0     |            |                  |              |      |       |      |
| 3ALEv1  | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 3ALEv2  | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 3ALEv3  | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 3AN1    | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 3AN2    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 3AN3    | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 3SC1    | Pseudomonas | γ- proteobacteria| 0     | 1          |                  |              |      |       |      |
| 3SC2    | Pseudomonas | γ- proteobacteria| 0     | 0          |                  |              |      |       |      |
| 3SC3    | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| 4ALEv1  | Pseudomonas | γ- proteobacteria| 0     |            |                  |              |      |       |      |
| Isolate | Genus         | Taxonomic Class | intI1 | Mutations* | intI1 pseudogene | qacE/qacE 1 | sul1 | ISCR1 | tniC |
|---------|---------------|-----------------|-------|------------|-----------------|-------------|------|-------|------|
| 4SC2    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 4SC3    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 4SL1    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 4SL2    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 4SLev1  | Pseudomonas   | γ- proteobacteria | 0     | 1          | 1               | 0           | 0    | 0     | 0    |
| 4SLev2  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 4SN1    | Pseudomonas   | γ- proteobacteria | 1     | 1          | 0               | 0           | 0    | 0     | 0    |
| 4SN2    | Burkholderia  | β- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 5ALev1  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 5ALev2  | Janthinobacterium | β- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 5AN1    | Flavobacterium | Flavobacteria    | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 5AN2    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 5AN3    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 6AL1    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 6AL2    | Serratia      | γ- proteobacteria | 0     | 1          | 0               | 0           | 0    | 0     | 0    |
| 6AL3    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 6AN1    | Serratia      | γ- proteobacteria | 0     | 1          | 1               | 0           | 0    | 0     | 0    |
| 7Alev1  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 7Alev2  | Chryseomonas  | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 7AN1    | Serratia      | γ- proteobacteria | 1     | 1          | 0               | 0           | 0    | 0     | 0    |
| 7AN2    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 1    | 0     | 0    |
| 7AN3    | Chryseomonas  | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 8ABC5A1 | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 8Alev1  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 8Alev2  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 8Alev3  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 8AN1    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 8AN2    | Janthinobacterium | β- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 9Alev1  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 9Alev2  | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 9AN1    | Pseudomonas   | γ- proteobacteria | 0     | 1          | 0               | 0           | 0    | 0     | 0    |
| 9AN2    | Burkholderia  | β- proteobacteria | 0     | 1          | 1               | 0           | 0    | 0     | 0    |
| 9AN3    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 9AN4    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 9AN5    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 9SC1    | Burkholderia  | β- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 9SN1    | Pseudomonas   | γ- proteobacteria | 1     | 4          | 0               | 0           | 0    | 0     | 0    |
| 9SN2    | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 9AL34   | Aranicola     | γ- proteobacteria | 1     | 9          | 0               | 0           | 1    | 0     | 0    |
| 10AL1   | Enterobacter  | γ- proteobacteria | 1     | 5          | 0               | 0           | 0    | 0     | 0    |
| 10AL3   | Serratia      | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 10AL4   | Pseudomonas   | γ- proteobacteria | 0     | 0          | 1               | 0           | 0    | 0     | 0    |
| 10AN2   | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 10SL1   | Pseudomonas   | γ- proteobacteria | 0     | 0          | 0               | 0           | 0    | 0     | 0    |
| 10SL2   | Pseudomonas   | γ- proteobacteria | 1     | 5          | 0               | 0           | 1    | 1     | 0    |
| 10SL3   | Pseudomonas   | γ- proteobacteria | 1     | 2          | 0               | 0           | 1    | 0     | 0    |
| 10SL4   | Pseudomonas   | γ- proteobacteria | 1     | 1          | 0               | 0           | 1    | 0     | 0    |
sul1 alleles of non-clinical strains that could be in turn maintained by the presence of contaminants that co-select for sulphonamide resistance.

A Wide Dissemination of the qacE1/qacED1 Gene was Found Between Taxa and Between Different Degrees of Urbanization in Patagonia

The distribution of the other ARG in this study, the qacE1/qacED1 gene, was wider among the environmental samples than found for sul1. Although we found that the qacE1/qacED1 gene was present in 28 out of 98 isolates and in 12 bacterial genera, this gene was not significantly related to “clean” or to urban sites (p>0.05) (Figure 3). We sequenced all qacE1/qacED1 amplicons, which exhibited 100% identity in 241 bp to the clinical allele (accession number HM999792). The high frequency of qac gene cassettes (qacE, qacG and qacH) found in non-clinical samples from Australia [51] allowed the authors to propose a relevant role for the qacE1 gene cassette in the origin of the most common genetic platform of the clinical class 1 integrons. Following their hypothesis, the common ancestor of class 1 integrons was embedded in a Tns402-like transposon harbouring a complete qacE1 gene cassette within the VR-1; this gene was subsequently deleted by insertion of the sul1 gene and converted into the well-known qacED1 of the clinical 3′-CS of class 1 integrons [1,52,53]. Recently, an environmental permafrost strain which was presumed to date from to 15,000–40,000 years ago with a typical 3′-CS of clinical class 1 integrons was found in Siberia [54]. If this finding is not a contamination, it is likely that the 3′-CS has been in nature and probably maintained without interaction with human activities before the antibiotic era.

Clearly, no matter which genetic platform contains the qacE1/qacED1 gene, its dissemination as either a cassette or a pseudogene between clinical and natural communities is widespread around the world since a long time ago [1,51,53,54] [this work], emphasizing the adaptive role that it possess for a large variety of genomes, habitats and possibly different types of stressors.

Multiple Interactions Define the Ecological and Molecular Behaviour of Each ARG

While we found that the sul1 and qacE1/qacED1 genes were usually located separately in our non-clinical isolates, both ARGs are embedded in the widespread 3′-CS of class 1 integrons when they are detected in clinical samples [1,53]. We found both ARG together in only one isolate, 1AL4, which corresponds to Streptomyces spp. (phylum: Actinobacteria). The sequence revealed 100% identity over 622 bp with the array of the 3′-CS (accession number EU118148). This finding is probably the result of the flow of clinical strains harbouring class 1 integrons with the typical 3′-CS from the hospital to site 1. It is likely that a lateral genetic transfer event to the strain 1AL4 Actinobacteria has happened since this species is rarely isolated from human infections.

Previous reports have shown that sulphonamide and quaternary ammonium compound resistances are usually found in bacterial isolates from natural communities [40,51,55,56,57]. Also, very relevant for the evolution of multidrug isolates, both ARGs have been identified as possessing the potential to co-select for multidrug resistance in non-clinical and clinical samples [40,56,57,58,59].

However, both ARGs differ from functional, taxonomic distribution and molecular perspectives when they are analysed separately. It is well known that the qacE1 gene is a mobile element since it has all of the features of a gene cassette, whereas the sul1 is an open reading frame not associated with an attC site. Thus, it is likely that the mobility conferred by the system’s integron/cassette could be one reason for the widespread dissemination of qacE1 within natural communities and genomes. The qacE1/qacED1 genes from our non-clinical samples were 100% identical to the 3′-CS of clinical class 1 integrons, showing a different molecular pattern to the sul1 gene. For the latter gene it is possible to distinguish its “clinical” or “environmental” origin on the basis of the different nucleotide sequences, as also shown for alleles of intI1.

The results of this study showed that the sul1 and qacE1/qacED1 genes have a different distribution between sites with different degrees of urbanization in Patagonia and diverse behaviour from a molecular perspective, suggesting that multiple interactions define the abundance of each type of ARG at a particular site. So these factors need to be analysed in individual studies for each antimicrobial resistance gene.

Non-clinical Samples from Patagonia are a Reservoir of ISCR1

The ISCR1 gene was found in γ and β-proteobacteria and in Actinobacteria isolates (n = 11) (Table 2), and it was common in Pseudomonasaceae (8 out of 11 positive isolates), which is the first description of this site-specific recombinase gene in non-clinical samples. The sequence of 475 bp from the 11 ISCR1-positive isolates revealed 100% identity with the clinical allele (EU722351). In a previous study from our laboratory on clinical isolates, this gene was present in 40% of 130 Enterobacteriaceae strains and only in 1% of 100 Pseudomonas aeruginosa isolates (data not shown), suggesting a different taxonomic distribution between clinical isolates compared to natural communities. The high frequency exhibited by ISCR1, as well as its distribution in several taxa in Patagonian samples, could be evidence of the important role of the open environment as a reservoir of this gene in our geographical region. The ISCR1 gene was found in bacterial cells in the same...
sampling sites where isolates with \textit{intI1}, \textit{qacE}1/\textit{qacE}\Delta1 and/or \textit{sul1} were also identified that ensures an encounter among cells and a putative transference of genes. This pool of genes could be the source for the emergence of the first strain harbouring \textit{bla}_{CTX-M-2} associated with \textit{ISCR1} on the genetic platform of a complex class 1 integron that had emerged in a clinical isolate in Argentina in 1989 [31,60].

**Abundance and Flow of the \textit{intI1} Gene in Environmental Samples**

We found a frequency of 11.2% \textit{intI1} genes in 98 isolates by plating on nutritive agar without antibiotics in 5 out of the 10 sampling sites. This frequency is relatively high in comparison to those obtained in previous studies. Rosewarne et al. [42] found only 0.5% of positive strains (4/790 isolates) and Stokes et al. [1] found 2.1% (4/192 isolates) in a similar model of a culture-based isolation of non-clinical strains without antibiotics in Australia. These contrasting results could be the consequence of different methodologies of isolation, types of habitats, bacterial communities, and also phylogenetic patterns could be involved in the abundance of this gene in different geographic regions. When we analysed the molecular features of the \textit{intI1} genes from our study, we identified two “clinical” genes of the \textit{intI1} gene that were found in the sampling site with the highest level of urbanization (site 1, Ushuaia city). These two “clinical” \textit{intI1} genes could belong to different clinical strains since they were different alleles obtained from different species of bacteria: sample 1SL5 was from a \textit{Pseudomonas} spp. (accession number DQ247972 with 22.13% of \textit{intI1} genes from Genbank) and sample 1AC2 was from an \textit{Aeromonas} spp. (CP000650 with 2.55% of \textit{intI1} genes from Genbank). The remaining nine alleles, which all were “environmental” \textit{intI1} genes (n = 5 in \textit{Pseudomonas} spp., n = 1 in \textit{Vibrio} spp. and n = 3 in \textit{Enterobacteriaceae}) (Table 2), showed sequence diversity with novel mutations that have never been described before; neither in non-clinical nor in clinical \textit{intI1} genes (Figure 4).

The \textit{intI1} gene flow from the hospital to the open environment has been well established [47,48,50]. Based on the large number of \textit{intI1} alleles found in non-clinical samples [1,48][this study], in clinical isolates (from GenBank, up until August 2010, Figure 4), and also found in both types of environments (Figure 4), we propose that at least two different routes of the acquisition of class 1 integrons could have interacting in hospitals during the antibiotic era: on one hand, the most common alleles of \textit{intI1} (accession numbers DQ247972, AY463797, AM412236 and DQ315789) must have been the first that were introduced in the hospital niche and, thereafter, were continuously selected by the pressure of antibiotics; and, on the other hand, in certain circumstances, “environmental” \textit{intI1} genes must have been taken from natural communities and thereby began their propagation and circulation in the clinical habitat under antimicrobial pressure.

In our understanding, this flow of genes from the open environment to hospitals is also evidenced by the new and unusual 3’-CS. In fact, this latest genetic platform has been described as harbouring the \textit{qacE1} gene cassette within the VR-1, which has been detected very frequently in non-clinical samples [26,51]. The high frequency of different \textit{intI1} alleles found in clinical and non-clinical isolates shown in the phylogenetic tree of the Figure 4 also suggests that \textit{intI1} gene flow between human activities and the environment occurs in both directions.

**The High Frequency of \textit{intI1} Pseudogenes Reveals a Similar Genomic and Ecological Behaviour for Integron Integrase**

The \textit{intI1} pseudogenes were found in 9 out of the 10 sampling sites of Tierra del Fuego Island. From a total of 30 \textit{intI1}-positive isolates, the frequency of occurrence of \textit{intI1} genes was 11.2% (11/98) if the entire \textit{intI1} gene sequence is considered, and it was 19.4% (19/98) if only the amplification of the integron integrase motif [62] is taken into account (Table 2). While the complete sequence of the \textit{intI1} gene was only detected in \gamma-protobacteria, the \textit{intI1} pseudogenes were also found in two \beta-protobacteria isolates and in one Actinobacteria isolate (ISC3). Thus, the range of dispersion of \textit{intI1} genes was increased between different taxa and between different sampling sites due to the identification of \textit{intI1} pseudogenes. However, this genetic marker was not related to urbanization, which demonstrates that the entire \textit{intI1} gene and its pseudogenes exhibit a similar ecological behaviour.

A previous bioinformatics study of all families of integron integrase genes found that 1/3 of \textit{intI1} were pseudogenes [63]. The prevalence of \textit{intI1} pseudogenes in our non-clinical samples was
two times higher than that of the entire intI1 gene. This greater frequency of intI1 pseudogenes observed in this study is evidence of the significant adverse effects produced by the entire gene in many different bacterial genomes. On the other hand, this widespread dissemination also highlights the fact that class 1 integrons possess a successful mechanism for spreading among natural bacterial communities. In addition, the intI1 pseudogenes have a different pattern of distribution if we compare the natural with the clinical communities. The bioinformatics study we performed on DNA sequences from clinical strains in Genbank (up until August 2010) revealed that only Corynebacterium diphtheriae (accession number BX248353) has an intI1 pseudogene. Thus, the low frequency of intI1 pseudogenes and, therefore, the high frequency of entire intI1 genes in the clinical isolates from GenBank revealed that the genomes circulating in clinical communities have possibly been selected because they have a genomic plasticity that facilitates maintenance of the entire intI1 gene.

The intI1 Genes Isolated from the Open Environment were not Related to Urbanization

Although a weak trend towards a high occurrence of intI1 genes in urban areas was observed, there were no statistical correlations

| Table 3. Wald statistic values obtained from the generalised lineal model analyses that were applied to the relationships between gene frequencies and 3 independent variables. |
|-----------------------------------------------|
|           | intI1 | intI1 mutations | intI1 pseudogen | tniC/ISCR1 | sul1 | qacE |
| Intercept | 0.01  | 10.59          | 1.35            | 0.01       | 0.01 | 1.80 |
| Substrate | 2.09  | 1.94           | 0.12            | 0.79       | 0.76 | 0.05 |
| Taxa      | 0.01  | 0.01           | 0.17            | 0.01       | 0.01 | 2.94 |
| Urbanization | 0.01 | 0.01          | 0.70            | 0.01       | 0.01 | 7.55* |

*represents p<0.05.
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between the mean occurrence per site of the intI1 gene and its pseudogenes and the three degrees of urbanization (p<0.05) (Figure 3; Table 3). In other words, “environmental” intI1 genes were not significantly more abundant in anthropic environments than in remote areas from urban centres.

However, previous reports showed that sites close to human activities have a higher frequency of intI1 genes, as a result of the intI1 flow from clinical samples to the open environment [92,64]. The discharge of genes should be maintained by the release of antibiotics at urban sites or by the presence of contaminants as metals [37,42,64]. The process of co-selection, would be involved in the maintenance of intI1 genes [42,47], as it is the case for transposon Tn21, which possesses determinants of resistance to mercury and usually has a class 1 integron embedded in its genetic platform [65]. Several studies evidenced the flow of class 1 integrons from humans to wastewater treatment plants, rivers, soil and domestic and wild animals [64,66,67,68,69,70,71,72]. From a molecular perspective, a similar scenario was found in our study, since the “clinical” alleles of the intI1 gene were only found in sites with a high level of urbanization.

When class 1 integrons were searched for in E. coli strains isolated from several animal populations subjected to different degrees of anthropogenic disturbance, the abundance of intI1 was found to correlate with the closeness to humans [64]. Skurnik et al. [64] explained the absence of class 1 integrons in wild animals as a result of never having been exposed to humans. However, the absence of class 1 integrons in E. coli strains not exposed to human disturbances can be expected since it has been suggested that the genome of E. coli is not able to acquire or maintain class 1 integrons without antibiotic pressure [55]. In other study, Rosewarne et al. (2010) [42] compared the abundance of intI1 in catchments with different levels of human disturbance from the Greater Melbourne area of Victoria, Australia, and found a strong positive relationship between the frequency of occurrence of this gene and heavy metal pollution. One explanation for our findings is that the apparently “clean” sites of Tierra del Fuego Island in fact receive or have received some source of pollution (flow of intI1-positive clinical strains, antibiotics and/or heavy metals) that is not associated with the level of urbanization. Some sites are visited by tourists, but most only have a very low frequency of visitors in the months when there is no snow. Nor is it likely to be explained by current pollution levels from heavy metals, which are concentrated in areas close to the cities in this region [73,74]. Moreover, the existence of pollution sources in the past is unlikely as the island of Tierra del Fuego has remained largely untapped for decades because of its geographical position and difficulty of access. Therefore, although cases of contamination could have been sporadic in the past, these could not explain the persistence of intI1 in non-urban regions. Another interpretation for our results is that the lack of correlation found in our study could be due to the small sample size; however, the frequency of occurrence of intI1 in “clean” sites of Tierra del Fuego Island was sufficiently large to deserve attention. Another hypothesis for explaining the differences between studies regarding the incidence of intI1 in “clean” sites could be that there are regions of the Earth in which some lateral genetic transfer mechanisms might be more abundant, due to historical processes of regional scale. Independent phylgenetic processes may have caused the intI1 gene to be differentially adapted to different bacterial genomes. The consequence would be that, in certain regions, bacterial species maintain the intI1 gene irrespective of anthropogenic pressure. These type of analysis, based on molecular and ecological studies but on a global scale, could be helpful for disentangling the multiple factors that are involved in the flow and maintenance of the intI1 gene between areas of human activities and natural communities.

Conclusions

Simultaneous analyses at ecological and molecular levels appeared to be a successful strategy for elucidating the role of each component of the genetic platforms associated to antimicrobial resistance of class 1 integrons.

We found that both ARGs (sul1 and qacE1/qacEΔ1 genes), which are usually embedded in the most common genetic platform of class 1 integrons within clinical habitats, showed different ecological and molecular behaviours in natural communities. While the presence of the sul1 gene was the only component of the genetic platforms of class 1 integrons related to urbanization, the qacE1/qacEΔ1 gene was found to be widespread in natural communities with different degrees of anthropogenic disturbances, which highlights the adaptive role of this gene to several different habitats. The LGE (intI1, ISCR1 and tniC genes) can exhibit high levels of diversity and different levels of persistence depending on the habitat and regions of the world. Ecological analysis showed that the intI1 gene, as well as the ISCR1 genes, which are relevant mechanisms involved in the spreading of multidrug resistance mechanisms in clinical isolates in Argentina and worldwide, were not associated with urbanization in the Patagonian samples. A total of 30/98 intI1-positive isolates were identified, with a high frequency of intI1 pseudogenes (19/98), which suggests that although intI1 has a deleterious impact within several genomes, it can easily be disseminated throughout natural bacterial communities. We cannot rule out the possibility that the high percentage of intI1 and ISCR1 genes that we found in the natural communities may be one of the factors that contributes to the increasing frequency of antimicrobial resistance isolates that have characterized Argentina for decades.

The main conclusion of this study is that the ability of natural bacterial communities to act as a reservoir and source of multidrug resistance mechanisms cannot be described by a general process but depend on multiple factors operating at molecular, ecological, phylgenetic and historical levels.

Materials and Methods

Study Area

The study was conducted in the south-eastern portion of Tierra del Fuego Island (Figure 2). The area lies within the Sub-Antarctic Deciduous Beech Forest, which is characterized by two species of southern beech, Nothofagus pumilio (Lenga) and Nothofagus betuloides (Guindo) [73]. Its climate belongs to the sub-polar oceanic type. Temperatures are cold all year round, with an average annual temperature of 5.7°C and low annual temperature variations, ranging from −0.3°C in July to 9.4°C in January. There are two urban sites: Ushuaia city with 80,000 inhabitants on the southern coast of the island, bathed in the Beagle Channel, and Tolhuin, a town of about 8000 inhabitants. The island was only colonized at the end of the nineteenth century.

All necessary permits for the described field studies were obtained from Gtitilde;itte Lizaralde (Director of the Planning Department in the science and technology area of the province) and Laura Malmierca (Tierra del Fuego National Park Management).

Definition of the Degree of Urbanization

Samples were collected in 10 sites that were selected according to three distinct levels of anthropogenic disturbance (Figure 2 and Table 1). The degree of urbanization was quantitatively
estimated by counting the number of buildings and roads that were contained in a 1 km circular area around each site. These estimations were conducted using satellite images provided by Google Earth. We defined a high level of urbanization as being when the number of buildings was greater than 50 and the number of routes greater than 5. We defined a low level of urbanization as being when the number of buildings was less than 5 and the number of routes less than 2. Two sites were located in Ushuaia City, in the mouths of the Pipo (Site 1) and Ushuaia (Site 10) rivers; three sites were in Tierra del Fuego National Park, at a stream that ends in Ensenada Bay (Site 7), at an upper portion of Pipo River (Site 8) and at Ovando River (Site 6); two sites were at Escondido Lake, one on the shore of the lake where a hotel is located (Site 4) and the other one at a stream that finishes in this lake (Site 5); two sites were along the Provincial Road N° 26 in a mountainous area south-east of Fagnano Lake, one of which was in the intersection of this road with Turbio River (Site 2) while the other was at Turbera Maucaisen (Site 3); and Site 9 was located in Moat Farm, in a stream close to the sea (Figure 2). Sites 3 to 9 had no history of clinical or industrial activities. Animal husbandry in the studied areas is minimal, and there is no systematic records of the use of antibiotics on domestic animals. The sites of Turbio River and Moat Farm are occasionally visited, mainly in the summer. Sites 1 and 10 were categorized as being highly disturbed, sites 3 and 4 were categorized as being medium-level disturbed, and the others were categorized as being low-level disturbed sites (Table 1).

**Sampling Techniques**

Samples were collected at each site between 20th January and 7th February 2006. Shallow freshwater sediment and soil samples from the shore were plated on nutritive agar medium (Britania, Argentina) without a selection of antibiotics. The plates were incubated at 4°C for 8 days, after which all individual colonies from each site and from each plate were plated again in nutritive agar and incubated at 4°C for 4 days. Then, each colony was picked out and place onto Luria Bertani broth and incubated at 4°C for 48 h.

**Molecular Analysis**

Because the goal of this work was to analyse the genetic platforms of class 1 integrons, we worked with a culture-dependent technique in order to identify how many ARG or LGE could be harboured in a single strain. The isolates were identified using standard biochemical tests, microbiological test strips (API20NE-Biomerieux, France) and sequencing of 16 S RNA using universal primers [76]: *Arthrobacter* spp. (n = 6), *Aeromonas* spp. (n = 4), *Vibrio* spp. (n = 2), *Enterobacter* spp. (n = 2), *Streptomyces* spp. (n = 1), *Microbacterium* spp. (n = 1), *Pseudomonas* spp. (n = 56), *Micrococcus* spp. (n = 1), *Yeastino bacterium* spp. (n = 7), *Yersinia* spp. (n = 2), *Flavobacterium* spp. (n = 3), *Pseudobacillus* spp. (n = 1), *Serratia* spp. (n = 5), *Burkholderia* spp. (n = 4), *Chryseomonas* spp. (n = 2), *Aranzola* spp. (n = 1).

Then, total DNA was extracted and Polymerase Chain Reaction (PCR) amplifications were carried out in 50 µl volumes containing 10 ng of DNA, 1 x PCR buffer (Promega, USA), 0.2 mM of dNTPs mix (Genbiotech, Argentina), 0.4 µM of each primer (Genbiotech, Argentina) and sterile distilled water, and *Tag* DNA polymerase (Promega, USA) was added (0.25 U). For the detection of the *intI1* gene, two strategies were used by amplifying a PCR fragment of 925-bp length (5’-ctgaccgtgctgaattc-3’ and 5’-ttggaatgtgactaatgac-3’) [32] and another of 483-bp length (5’-acatcggaaatagtccgta-3’ and 5’-gctggttgaatgtgctga-3’) [77] that included the additional motif that it is conserved among integron integrases [62,78,79]. When only the 483-bp amplicon was obtained, the HS913 primer (5’-ctgaccgtaaatccag-c-3’) in conjunction with the HS916 primer (5’-ttggaatgtgactaatgac-3’) [90] was used in order to detect a putative, whole *intI1* gene. The detection of *intII* and *intI1* pseudogenes was performed by two people at different times with independent DNA extractions and repeated at least twice. Also, the presence of *sulI* (5’-ttggaatgtgactaatgac-3’ and 5’-gctggttgaatgtgctga-3’) [81], *queE1/queE2* (5’-ctgaccgtgctgaattc-3’ and 5’-agcccaacctcggc-3’) [30], *ISCR1* (5’-attggaatgtgactaatgac-3’ and 5’-ctgaccgtgctgaattc-3’) [32] and *tnoC* (5’-ctgaccgtgctgaattc-3’ and 5’-gctggttgaatgtgctga-3’) genes were investigated in all strains. The PCR products were sequenced after purification using the Wizard SV Gel and PCR clean-up System Kit according to the manufacturer’s directions (Promega, USA); sequencing was performed on both DNA strands using ABI Prism 3100 BioAnalyzer equipment (Applied Biosystems, USA). The nucleotide sequences were analysed using Genetics Computer Group (GCG) and Blast V2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/).

**Definition of “Clinical” and “Environmental” *intI1* Alleles**

We called non-clinical *intI1* genes those that were harboured by the strains isolated from water, sediment or soil in this work. The non-clinical *intI1* gene can also be an “environmental” or a “clinical” allele as defined by Gillings et al., suggesting a putative source from natural or clinical communities, respectively [48].

**Bioinformatics Study of the *intI1* Gene**

We identified 47 alleles from clinical samples in Genbank (up until August 2010); the most common alleles used for defining a “clinical” allele were those with the accession numbers DQ247972 (22.13%),AY463797 (19.15%), AM412236 (18.72%) and DQ915789 (17.02%).

**Phylogenetic Analysis**

Phylogenetic evolutionary analysis for *intI1* and *sulI* sequences were conducted using MEGA v 5.05 software [82]. Sequences obtained in this study as well as alleles deposited in GenBank were included. These sequences were aligned using ClustalW application in MEGA with default parameters. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps were eliminated.

**Statistical Analysis**

The relationship between the environmental characteristics and the distribution of molecular components of antibiotic resistance were analysed using two statistical approaches (STATISTICA package). Generalized linear models (GLM) were applied with the following characteristics: (1) dependent variable: presence/absence of *intI1*, *sulI*, *tnoC* and *ISCR1*, and number of *intI1* mutations; (2) independent variables: two categorical variables, substrate (water or soil) and taxa (β-proteobacteria or other taxa) and one ordinal variable, degree of urbanization (1, 2 or 3); (3) assumed distribution of the dependent variable: binomial for presence/absence data and ordinal multinomial for mutations; (4) link function: logit.

The GLM was complemented with classical non-parametric tests. For the role of substrate and taxa in the presence of resistance genes, we used 2 x 2 contingency tables and Fisher’s exact tests, applied to frequencies of occurrence. For the effect of degree of urbanization, we applied Spearman’s rank correlations to mean occurrences of genes per site as a dependent variable.

Plasmid DNA was isolated from each site using a cetyltrimethylammonium bromide lysis buffer [83] and the plasmid band was fractionated by agarose gel electrophoresis, stained with ethidium bromide and detected by UV transillumination.
Nucleotide Sequence Accession Numbers

InfI and sfl sequences were deposited at GenBank as accession numbers JN870902 to JN870912 and JX048395 to JX048604 respectively.

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Author Contributions

Conceived and designed the experiments: DC. Performed the experiments: MN. Analyzed the data: DC. Contributed reagents/materials/analysis tools: DC. Wrote the paper: DC. M.H.C. M.S.R.

References

1. Stokes HW, Nohno CL, Holley M, Bahl MI, Gillings MR, et al. (2006) Class 1 integrons potentially predating the association with Tra62-like transposition genes are present in a sediment microbial community. J Bacteriol 188: 5722–5730.

2. Martínez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321: 365–367.

3. Singer RS, Hoferce CL (2006) Potential impacts of antibiotic use in poultry production. Avian Dis 50: 161–172.

4. Baquero F, Martínez JL, Cantoñ R, Cantoña R (2008) Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 19: 260–265.

5. Macinga DR, Rather PN (1999) The chromosomal aminoglycoside 2”-N-acytelytransferase of Providencia stuartii: physiological functions and genetic regulation. Front Biosci 4: D132–140.

6. Franklin K, Clarke AJ (2001) Overexpression and characterization of the chromosomal aminoglycoside 2”-N-acytelytransferase of Providencia stuartii. Antimiicrob Agents Chemother 45: 2238–2244.

7. Walsh TR (2006) Combinatorial genetic evolution of multiresistance. Curr Opin Biotechnol 19: 261–265.

8. Holmes AJ, Gillings MR, Nield BS, Mabbutt BC, Nevalainen KM, et al. (2003) Molecular epidemiology of aVI1-class-1 integrons in multidrug resistant isolates from Argentinean hospitals. Antimiicrob Agents Chemother 47: 3945–3949.

9. Holmes AJ, Roy PH, Jacoby GA, Orman BE, Piteño SA, et al. (2002) blaCTX-M-14 is located in an unusual class 1 integron (In35) which includes Oxy1. Antimiicrob Agents Chemother 46: 2303–2308.

10. Quiroga MP, Andres P, Petroni A, Soler Bistue AJ, Guerriero L, et al. (2007) Complex class 1 integrons with diverse variable regions, including aa(67)’Bcr-U, and a novel allele, aminB10, associated with ISGRI in clinical enterobacterial isolates from Argentina. Antimiicrob Agents Chemother 51: 4460–4470.

11. Kholodii GY, Mindlin SZ, Bass IA, Yurieva OV, Minakhina SV, et al. (1995) Four genes, two ends, and a σ transition region are involved in transposition of Tn5033: a paradigm for a novel family of transposons carrying either a σ operon or an integrase. Mol Microbiol 17: 1189–1200.

12. Stiles DH, Abramson JJ, Hoferce CL, Summers AO (2004) Gram-positive bacteria are an unusual In0-like class 1 integron containing CR1 and blacTX-M2 and associated with a Tra62-like element. Antimiicrob Agents Chemother 50: 799–802.

13. Holmes AJ, Gillings MR, Hughes L, Andrew NR, Nield BS, et al. (2001) Molecular epidemiology of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exoticus. Antimiicrob Agents Chemother 45: 723–726.

14. Barlow RS, Pemberton JM, Desmarchelier PM, Gibbons KS (2004) Identification and characterization of integron-containing bacteria without antibiotic selection. Antimiicrob Agents Chemother 48: 4317–3425.

15. Ehrlich SA, Stokes HW, Findlay S, Taylor M, Gillings MR (2008) Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. FEMS Microbiol Lett 278: 267–272.

16. Kollodiy GV, Miniflin SZ, Bass IA, Yurieva OV, Minakhina SV, et al. (1995) Four genes, two ends, and a σ transition region are involved in transposition of Tn5033: a paradigm for a novel family of transposons carrying either a σ operon or an integrase. Mol Microbiol 17: 1189–1200.

17. Rozen CA, Cobb CL, Hughes L, Andrew NR, Nield BS, et al. (2001) Incidence and characterization of integron-containing bacteria without antibiotic selection. Antimiicrob Agents Chemother 45: 4229–4232.

18. Stiles DH, Abramson JJ, Hoferce CL, Summers AO (2004) Gram-positive bacteria are an unusual In0-like class 1 integron containing CR1 and blacTX-M2 and associated with a Tra62-like element. Antimiicrob Agents Chemother 50: 799–802.

19. Holmes AJ, Roy PH, Jacoby GA, Orman BE, Piteño SA, et al. (2002) blaCTX-M-14 is located in an unusual class 1 integron (In35) which includes Oxy1. Antimiicrob Agents Chemother 46: 2303–2308.

20. Quiroga MP, Andres P, Petroni A, Soler Bistue AJ, Guerriero L, et al. (2007) Complex class 1 integrons with diverse variable regions, including aa(67)’Bcr-U, and a novel allele, aminB10, associated with ISGRI in clinical enterobacterial isolates from Argentina. Antimiicrob Agents Chemother 51: 4460–4470.

21. Kollodiy GV, Miniflin SZ, Bass IA, Yurieva OV, Minakhina SV, et al. (1995) Four genes, two ends, and a σ transition region are involved in transposition of Tn5033: a paradigm for a novel family of transposons carrying either a σ operon or an integrase. Mol Microbiol 17: 1189–1200.

22. Rozen CA, Cobb CL, Hughes L, Andrew NR, Nield BS, et al. (2001) Incidence and characterization of integron-containing bacteria without antibiotic selection. Antimiicrob Agents Chemother 45: 4229–4232.
50. Stokes HW, Gillings MR (2011) Gene flow, mobile genetic elements and the evolution of class 1 integrons. J Bacteriol 193: 5095–5100.

51. Gillings MR, Holley MP, Stokes HW (2009) Evidence for dynamic exchange of gene cassettes between class 1 integrons and other integrons in freshwater biofilms. FEBS Microbiol Lett 296: 202–208.

52. Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, et al. (2008) The evolution of class 1 integrons and the rise of antibiotic resistance. J Bacteriol 190: 5093–5100.

53. Gillings MR, Xuejun D, Hardwick SA, Holley MP, Stokes HW (2009) Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? ISME J 3: 209–213.

54. Petrova M, Gorlenko Z, Mindlin S (2011) Tn53.

55. Díaz-Mejía JJ, Amabilé-Cuevas CF, Rosas I, Souza V (2008) An analysis of the dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese Salmo nella enterica strains and relation with integrons. Antimicrob Agents Chemother 49: 836–839.

56. Antunes P, Machado J, Sousa JC, Peixe L (2005) Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese Salmonella enterica strains and relation with integrons. Antimicrob Agents Chemother 49: 836–839.

57. Kaxama H, Hamashima H, Sasatsu M, Arat T (1998) Distribution of the antiseptic-resistance genes qacE and qacEA1 in gram-negative bacteria. FEBS Microbiol Lett 159: 173–178.

58. Paulsen IT, Littlejohn TG, Räderstrom P, Sundström I, Sköld O, et al. (1993) The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antisepsics and disinfectants. Antimicrob Agents Chemother 37: 761–768.

59. Räderstrom P, Swedberg G, Sköld O (1991) Genetic analyses of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. Antimicrob Agents Chemother 35: 1840–1848.

60. Bauerfeind A, Casellas JM, Goldberg M, Holley M, Jungwirth R, et al. (1992) A new plasmidic cefotaximase from patients infected with Salmonella enterica serotype typhimurium. J Antimicrob Chemother 57: 1215–1219.

61. Roy Chowdhury P, Ingold A, Vanegas N, Martínez E, Merlino J, et al. (2011) Dissemination of multiple drug resistance genes by class 1 integrons in Klebsiella pneumoniae isolates from four countries: a comparative study. Antimicrob Agents Chemother 55: 3140–3149.

62. Messier N, Roy PH (2001) Integron integrases possess a unique additional domain necessary for activity. J Bacteriol 183: 6699–6706.

63. Nemergut DR, Robeson MS, Kysela RF, Martin AF, Schmidt SK, et al. (2008) Insights and inferences about integron evolution from genomic data. BMC Genomics 9: 261.

64. Skurnik D, Ruimy R, Andremont A, Amorin C, Rouquet P, et al. (2006) Effect of human vicinity on antimicrobial resistance and integrons in animal faecal Escherichia coli. J Antimicrob Chemother 57: 1215–1219.

65. Liebert CA, Hall RM, Summers AO (1999) Transposon Tn21, flagship of the floating genome. Microbiol Mol Biol Rev 63: 507–522.

66. Laroche E, Pasdalk B, Berthe T, Skurnik D, Petit F (2009) Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in Escherichia coli isolated from a densely populated estuary (Seine, France). FEBS Microbiol Ecol 68: 118–130.

67. Laroche E, Petit E, Fournier M, Pasdalk B (2010) Transport of antibiotic-resistant Escherichia coli in a public rural karst water supply. Journal of Hydrology 392: 12–21.

68. Bonnedahl J, Olsen B, Waldenström J, Boman T, Jalava J, et al. (2008) Antibiotic susceptibility of faecal bacteria in Antarctic penguins. Polar Biology 31: 755–763.

69. Dolejska M, Bierosova B, Kohoutova L, Literak I, Cizek A (2009) Antibiotic-resistant Salmonella and Escherichia coli isolates with integrons and extended-spectrum beta-lactamas in surface water and sympatric black-headed gulls. J Appl Microbiol 106: 194–201.

70. Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, et al. (2010) Antimicrobial-resistant faecal Escherichia coli in wild mammals in central Europe: multiresistant Escherichia coli producing extended-spectrum beta-lactamas in wild bears. J Appl Microbiol 108: 1702–1711.

71. Bartoloni A, Pallecchi L, Rodriguez H, Fernandez C, Mantella A, et al. (2009) Antibiotic resistance in a very remote Amazonas community. Int J Antimicrob Agents 33: 125–129.

72. Schilter A, Szczepanowski R, Kurz N, Schneiker S, Krahn I, et al. (2007) Erythromycin resistance-confering plasmid pRS105, isolated from a sewage treatment plant, harbors a new macrolide resistance determinant, an integron-containing Tn926-like element, and a large region of unknown function. Appl Environ Microbiol 73: 1932–1960.

73. Amín O, Ferrer L, Maccovechio J (1996) Heavy metal concentrations in littoral sediments from the Beagle Channel, Tierra del Fuego, Argentina. Environ Monit Assess 41: 219–231.

74. Amín O, Conoglio L, Spetter C, Duarte C, Astrausain R, et al. (2011) Assessment of land influence on a high-latitude marine coastal system: Tierra del Fuego, southmost Argentina. Environ Monit Assess 175: 63–73.

75. Gutiérrez E, Vallejo VR, Romanýa J, Fons J (1991) The subantarctic Nothofagus forests of Tierra del Fuego: distribution, structure and production. Oecologia Aquatica 10: 351–366.

76. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.

77. Hoyle DV, Davison HC, Knight HI, Yates CM, Dohay O, et al. (2006) Molecular characterisation of bovine faecal Escherichia coli shows persistence of defined ampicillin resistant strains and the presence of class 1 integrons on an organic beef farm. Vet Microbiol 115: 250–257.

78. Gravel A, Messier N, Roy PH (1998) Point mutations in the integron integrase intI1 that affect recombination and/or substrate recognition. J Bacteriol 180: 5437–5442.

79. Nield BS, Holmes AJ, Gillings MR, Recchia GD, Mabbutt BC, et al. (2001) Recovery of new integron classes from environmental DNA. FEBS Microbiol Lett 195: 59–65.

80. Roy Chowdhury P, Merlino P, Labbate M, Cheong EY, Gontlieb T, et al. (2009) Tn5960, a transposon from a genomic island in a Pseudomonas aeruginosa clinical isolate that includes two class 1 integrons. Antimicrob Agents Chemother 53: 5294–5296.

81. Barbolla R, Catalano M, Orman BE, Famiglietti A, Vay C, et al. (2004) Class 1 integron in epidemiologically unrelated Stenotrophomonas maltophilia isolates. Antimicrob Agents Chemother 48: 660–669.

82. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolution- ary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.