REVIEW ARTICLE

Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens

Hatch W. Stokes[1] & Michael R. Gillings[2]

[1]The i3 Institute, University of Technology, Sydney, Broadway, NSW, Australia; and [2]Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia

Correspondence: H.W. Stokes, The i3 Institute, University of Technology, PO Box 123, Broadway 2007, Sydney, NSW, Australia. Tel.: +612 9514 2232; fax: +612 9514 8206; e-mail: hatch.stokes@uts.edu.au

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Abstract

Antibiotics were one of the great discoveries of the 20th century. However, resistance appeared even in the earliest years of the antibiotic era. Antibiotic resistance continues to become worse, despite the ever-increasing resources devoted to combat the problem. One of the most important factors in the development of resistance to antibiotics is the remarkable ability of bacteria to share genetic resources via Lateral Gene Transfer (LGT). LGT occurs on a global scale, such that in theory, any gene in any organism anywhere in the microbial biosphere might be mobilized and spread. With sufficiently strong selection, any gene may spread to a point where it establishes a global presence. From an antibiotic resistance perspective, this means that a resistance phenotype can appear in a diverse range of infections around the globe nearly simultaneously. We discuss the forces and agents that make this LGT possible and argue that the problem of resistance can ultimately only be managed by understanding the problem from a broad ecological and evolutionary perspective. We also argue that human activities are exacerbating the problem by increasing the tempo of LGT and bacterial evolution for many traits that are important to humans.

Introduction

Bacteria have methods of gene exchange that are distinct from those in eukaryotes, but they still conform to the laws of evolution by natural selection. When considering the antibiotic resistance problem, this fundamental point should not be forgotten (Antonovics *et al.*, 2007). Our real challenge is to understand the repertoire of processes and genetic elements that prokaryotes have available to them, and upon which natural selection can act. Lateral Gene Transfer, or more recently, Lateral *Genetic* Transfer (LGT) (Ragan & Beiko, 2009) is an important process in moving and rearranging DNA in prokaryotes. The extent of LGT is substantial, with estimates that up to 25% of some bacterial genomes can be derived from LGT over evolutionary periods of time (Ochman *et al.*, 2000). Even over much shorter time frames, the evolution of genomes by inheritance of large blocks of DNA from elsewhere can generate phenomenal amounts of diversity, particularly where selection is very strong. For instance, *Escherichia coli* can follow many different evolutionary paths based on DNA that is present in only some strains. This diversity is impressive, with the 'pan genome' of *E. coli* encompassing nearly 18 000 genes despite the fact that the coding content of a single cell of this species is slightly over 2000 genes (Touchon *et al.*, 2009).

The most overt example of evolution driven by selection is the selection for antibiotic resistance in pathogens. The dissemination of resistance genes is a direct consequence of LGT and this has enormous ramifications for human health. Indeed, it has been argued that the antibiotic/antibiotic resistance arms race is one that humans are losing (Falagas & Bliziotis, 2007). If so, one of the main reasons is that LGT potentially makes all genes in the microbial biosphere a single, common and shared resource. In the same way that wars can be won by nations with the greatest industrial capacity, so it is that bacteria can draw on a global resource that, with the means of LGT, can mobilize and transfer useful genes across physical and phylogenetic distances very rapidly. Unlike eukaryotes, therefore, bacteria are not dependent on random variation in genes within cell lines as templates on which natural selection can act. In existing and newly emerging pathogens, the survival and amplification of

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strains with enhanced pathogenicity may result from the acquisition of genes that evolved in an environment remote from humans and in a bacterium that is yet to be cultured. Managing infectious disease in the long term can only be achieved by understanding the basic concepts of evolutionary biology and how they apply to prokaryotes (Summers, 2002; Nesse & Stearns, 2008).

What is LGT?

The literature on LGT, also known as Horizontal Gene Transfer (HGT) (Frost *et al.*, 2005), is extensive. However, most of this literature focuses on the implications of LGT for the evolution of microbial genomes (Boto, 2010) or on issues relating to microbial phylogeny and the relevance of the species concept as it applies to prokaryotes (Bapteste *et al.*, 2009; Gribaldo & Brochier, 2009). Ironically, despite a focus on LGT and the arguments for its key role in microbial evolution, the concept itself is often poorly defined. It is beyond the scope of this review to fully describe the process, but some general issues need to be clearly understood. We define LGT as the process whereby DNA from one cell is physically transferred from one cell to another without an absolute requirement for cell division and the incorporation of that DNA into the recipient's genome such that it can be stably inherited. Without reflecting on a specific formal definition, this would be most people's understanding of the process. Thus, LGT requires at least two independent processes to occur: (1) physical movement of DNA and (2) incorporation into the receiving genome such as to allow stable inheritance (Table 1).

Each of these two steps can occur via a relatively small number of mechanisms. Physical movement occurs by one of the processes of conjugation, transduction or transformation and incorporation of DNA either by homologous or illegitimate recombination, transposition, site-specific recombination or by virtue of the transferred DNA being an independent replicon (Thomas & Nielsen, 2005). A successful transfer outcome requires the agency of at least one of the processes of movement and at least one of incorporation. The advent of very strong and near-global selection for antibiotic resistance has seen a huge increase in the number of cells carrying the corresponding resistance genes. This increase in the abundance of specific genes and their mobilizing elements allows more opportunity for the mechanisms of LGT to act cooperatively. Thus, mobile antibiotic resistance genes that make up parts of increasingly complex mosaic structures of identical or related sequences allow the capture of DNA by combinatorial exchange involving all of the factors listed in Table 1. Other features of LGT include the fact that it is both infectious – transfer can be associated with DNA replication resulting in a net increase of DNA per cell – and promiscuous – DNA transfer can occur across species. In environments uncorrupted by humans, these phenomena are probably more limited, in the case of promiscuity because barriers to trans-species DNA movement are known (Thomas & Nielsen, 2005). However, the strong selection being applied by the use of antibiotics is likewise seeing a breakdown of some of these natural limiting mechanisms.

Table 1. The collective forces that drive Lateral Gene Transfer

Mechanisms of transfer	Mechanisms of incorporation	Mobile elements*
Conjugation	1 – Autonomous replication	Plasmids† (1)
Transformation	2 – Transposition	Transposons (2)
Transduction	3 – Site-specific recombination	Insertion sequence common regions (2)
	4 – Homologous recombination	Integrative and conjugative elements† (3)
		Gene cassettes (3)
		Integrons‡

A successful LGT event requires the action of at least one transfer mechanism and one integration mechanism. All of the mobile elements named have the potential to move by any of the transfer mechanisms shown, although plasmids and integrative and conjugative elements most commonly transfer by conjugation.

*Numbers in brackets identify the major mechanism of incorporation. That is, the major process by which DNA achieves the ability to maintain itself in the receiving genome after physical transfer or uptake has taken place as described in the text.

†Plasmids and integrative and conjugative elements are also agents of gene transfer because they can move genes between cells by conjugation as well as integrate them.

‡Integrons lack the ability to integrate autonomously. However, the so-called *mobile* integrons have become associated with transposons and plasmids (or both). In these contexts, they piggyback on the functions of the associated element. With the acceleration of the evolution of mobile DNA and the appearance of multiple copies of similar, or identical elements in the same cell, homologous recombination can be considered an integrating mechanism for any of the elements listed.

Where do antibiotic resistance genes come from?

Antibiotics are not a human invention. Equally, antibiotic resistance genes did not evolve in bacterial pathogens as a defence against humans inventions. Rather, both antibiotics and the proteins that protect against them have a broad environmental origin (Martinez, 2008) that dates back millions and possibly billions of years (Baltz, 2008). The presence of antibiotics and of genes that confer resistance to them is an outcome of Darwinian selection in the microbial world. It has long been regarded that a major role of antibiotic production in natural environments is to allow niche exploitation on the part of those bacteria that produce them and that resistance is a selective response to such

production on the part of the producers themselves as well as their potential targets (Waksman & Woodruff, 1940). There is good evidence to support this hypothesis (Wiener, 1996), although it is also clear that antibiotic production (Davies *et al.*, 2006) and resistance may have other roles to play in natural environments, both at the community and at the cellular level (Groh *et al.*, 2007; Martinez, 2008). With the many roles that antibiotics play in the microbial biosphere, it has long been expected that antibiotic resistance genes and bacteria would be very common. In contemporary times, there is substantial evidence that this is true. Firstly, antibiotic resistance genes have been enriched and extensively mobilized through the widespread use of antibiotics by humans (this will be explored in detail later). Secondly, tools of the genomics era have provided a window into the diversity that has always existed in the microbial biosphere. Early studies predominantly found resistance genes reflective of those already found in clinical contexts (Benveniste & Davies, 1973) because they were most easy to select for and recover. In contrast, contemporary studies (D'Costa *et al.*, 2006), especially those using metagenomics approaches that encompass yet to be cultured bacteria, recover much more diverse resistance genes, including some that would not be readily be identifiable as such by bioinformatics analysis alone (Riesenfeld *et al.*, 2004; Allen *et al.*, 2009a). This has obvious ramifications for the management of antibiotic resistance because it implies that the pool of resistance genes that cause the current clinical problem may be the tip of a large iceberg. Compounding this problem is the fact that novel resistance determinants may already be undergoing mobilization by processes of LGT (Rowe-Magnus *et al.*, 2001; Nield *et al.*, 2004). The global nature of the resistance evolution problem is summarized in Fig. 1, whereby many low copy number resistance genes and mobilizing elements are distributed throughout the microbial biosphere. Over time (which is very short in the context of bacterial evolution), the recruitment of some resistance genes into some mobile elements has seen the subsequent introduction of both into pathogens. Once this began, further selection acted to accelerate this process, ultimately allowing the global spread and amplification of a small number of genes and mobile element types.

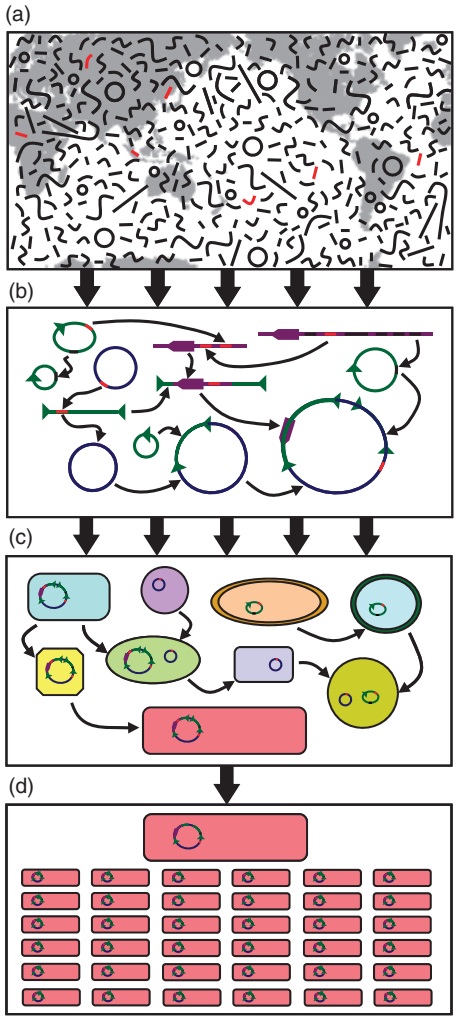

Fig. 1. Recruitment of resistance genes and mobilizing elements into pathogens. (a) Diagrammatic representation of the global distribution of mobile genes, mobilizable genes and mobilizing genetic elements as found in the preantibiotic era. (b) Pre- and postantibiotic era random rearrangements bring together mobilizing agents and genes encoding adaptive genes in niche environments. (c) Mobilizing genes move through microbial communities including human pathogens (in pink). (d) With strong selection as occurred in pathogens in the antibiotic era, selected organisms underwent clonal and global expansion.

Resistance determinants in vectors, bacterial species and animal hosts

The accumulation of antimicrobial resistance genes in pathogens has generated a world-wide crisis in the management of infectious disease (Davies, 2007). To control the spread of existing and novel resistance genes, we need an understanding of the genetic elements involved, of the dynamics of LTG and of microbial ecology (Salyers & Shoemaker, 2006). In turn, this requires the assembly of many different kinds of data, much of which we do not yet have.

What kinds of information might be required to understand the ecology of resistance and what is the most efficient way to approach the problem? The dynamics of resistance genes must be examined at a variety of different scales (Baquero, 2009), including the diversity of the genes themselves, the families of mosaic DNA elements that carry them, the bacterial strains and species in which they occur and the animals that host these bacteria (see Fig. 2). We also need to assess the probability that potential resistance mechanisms exist and the likelihood that genes encoding these

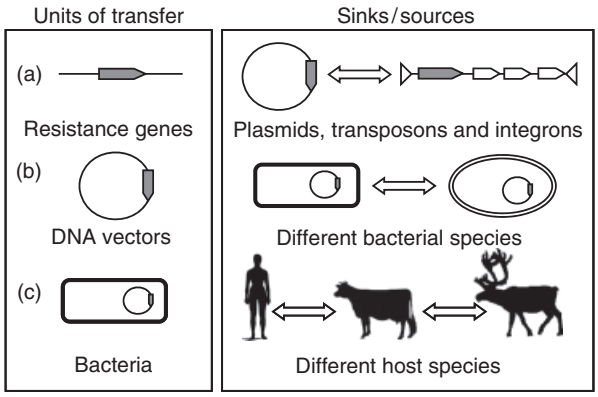

Fig. 2. Movement and mobilization of antibiotic resistance genes. The lateral transfer of resistance determinants can be examined from different perspectives: (a) Movement of individual resistance determinants can be mapped to particular chromosomal locations, plasmids, transposons or integrons. (b) Mobile vectors that carry these determinants can be tracked through different bacterial species. (c) Bacterial strains containing these vectors can be identified in different host animals. Lateral events can occur at any of these levels. For instance, a resistance gene may transpose from a chromosome to a plasmid, which then conjugates from an environmentally acquired bacterium into a commensal species residing in a new host. In each case, the lateral transfer can be recognized by genetic identity, but the direction of the transfer cannot be reliably ascribed without extensive temporal data.

mechanisms can successfully disseminate under conditions where selection will fix the recipients in populations (Martinez *et al.*, 2007). Above all, we need to move towards a broader evolutionary and ecological perspective on the problem (Aminov & Mackie, 2007).

Characterizing the units of transfer (Fig. 2a), particularly the resistance genes themselves, is an important first step, and one in which significant progress has been made. For instance, there have been two recent, comprehensive reviews on genes for β-lactamases, dealing with their diversity, mobility and epidemiology (Smet *et al.*, 2009; Poirel *et al.*, 2010). Similarly, the resistance genes encoded by integron gene cassettes have been systematically tabulated (Partridge *et al.*, 2009).

Compilations of resistance determinants generally deal with known genes characterized from known organisms. However, it is now widely accepted that culturable bacteria represent only a small fraction of bacterial genetic diversity, and consequently, the extent and diversity of resistance genes in the bacterial metagenome also need to be considered. Diverse, unusual β-lactamase genes and additional, novel genes encoding resistance to aminoglycosides and tetracycline have been readily recovered in metagenomic libraries generated from soil bacteria (Riesenfeld *et al.*, 2004; Allen *et al.*, 2009b). Other metagenomic studies of soil and marine sediments have demonstrated that the pool of novel gene cassettes available to integrons is vast and diverse (Michael *et al.*, 2004; Koenig *et al.*, 2008). While the phenotypes encoded by these environmental gene cassettes are for the most part unknown (Holmes *et al.*, 2003; Boucher *et al.*, 2007; Moura *et al.*, 2010), all are likely to be adaptive under some circumstances. Databases of integron gene cassettes and their associated recombination machinery are now available (Joss *et al.*, 2009; Moura *et al.*, 2009) and will continue to grow. There is also considerable interest in characterizing the antibiotic resistome more generally from environmental samples (D'Costa *et al.*, 2006; Wright, 2007), with a view to predicting novel resistance mechanisms and informing the rational development of new antibiotics. In the near future, the application of next-generation sequencing technologies will rapidly increase our catalogue of resistance genes.

The movement of resistance genes between physical locations is facilitated by a variety of genetic elements such as integrons, transposons, integrative conjugative elements (ICEs), plasmids and genomic islands, which are themselves also units of lateral transfer (Fig. 2b). Some of these elements are extraordinarily abundant. For example, transposases are ubiquitous and the most abundant genes in nature (Aziz *et al.*, 2010). For this reason, understanding the origins of such mobile elements is complex and is further complicated by the fact that mobile elements often form mosaics, built up from different genes and subelements, each with different evolutionary histories (Toussaint & Merlin, 2002; Norman *et al.*, 2009). The mosaic complexity of such molecules allows diverse interactions with other genetic elements, promoting exchanges that, in turn, generate more diversity (Garriss *et al.*, 2009; Wozniak & Waldor, 2010). A further complication arises in analyses of mobile genetic elements because they do not 'belong' to a particular cell or lineage and have independent evolutionary trajectories when compared to phylogenetic trees. One consequence of this independence is that genome sequencing of individual strains or species of Bacteria is not an efficient way to collect data on mobile elements (Frost *et al.*, 2005), although more efficient and cost-effective genome sequencing will progressively help this problem. Nonetheless, means of directly accessing the mobile gene pool need to be developed, and frameworks for consistent classification and nomenclature of mobile elements need to be used (Roberts *et al.*, 2008a; Leplae *et al.*, 2010).

Reservoirs of resistance?

What about the species of bacteria in which mobile vectors and resistance genes reside (Fig. 2c)? Most attention in the literature appears to be focused on characterizing pathogens by first identifying the species and the strain/clone involved and then examining the resistance genes contained therein. While this is a laudable exercise for particular clinical circumstances, if the phenotypes of interest (virulence,

resistance, etc.) are encoded on mobile DNA, then the DNA element is probably the factor of most importance, not necessarily the cellular background. Because of the selection pressure imposed by antibiotics, even transitory colonization by allochthonous bacteria offers ample opportunity for the fixation of rare lateral transfer events between species, and the movement of resistance genes and their vectors between animal hosts is not surprising. Therefore, if individual environments are considered in isolation, without taking into account the power of natural selection and LGT, any environment can appear to be a 'source' of resistance genes.

Naturally occurring antibiotic resistance genes have been a feature of microbial communities for a long time. Putting aside the question of what elements may be contributing to the LGT of resistance genes, one key question is: what do we know about the link between different environments in the microbial biosphere? Specifically, what environments can act as a source for the recruitment of antibiotic resistance genes into bacterial pathogens, especially those that cause nosocomial infections? This is a subject of interest for obvious reasons, and in recent years, many studies have identified a variety of environments and organisms that are potential 'reservoirs' of antibiotic resistance genes.

The references cited in Tables 2 and 3 were published in the interval of 2008–2010 and deal with the detection of one specific family of mobile elements – integrons – in animals and natural environments. Almost one-third of these publications (36 of 114) mention animals or environments as a 'reservoir' of antibiotic resistance. Slightly less than a third (33 of 114) use the more accurate term 'dissemination' to describe the movement of genes between genetic, biological and physical locations. The fact that the descriptor *reservoir* has come to be applied so ubiquitously is in itself an argument that the definition is inappropriate and that the concept is unhelpful. Rather, what the collective literature in this area reinforces is that, notwithstanding the qualitative and quantitative differences between environments, antibiotic resistance genes are a near-universal feature of the microbial biosphere. Although 'reservoir' may not be a good descriptor, we believe that *E. coli* (Bailey *et al.*, 2010) may be a particularly important contributor to the spread of antibiotic resistance genes globally. There are three reasons for this: (1) *E. coli* is an important animal commensal, (2) it can persist in the environment well away from an animal host and (3) strain variants of this species can commonly be pathogenic. To varying degrees, the same argument can be extended to other genera and species that meet these criteria (Salyers *et al.*, 2004).

Conduits of LGT

Most genes that are now mobile in clinical isolates probably originated as genes with a fixed chromosomal origin. Where this origin can be identified, it may be possible to ascribe putative origins to resistance genes or gene families as has been suggested for *qnr* genes (Poirel *et al.*, 2005). However, we argue that reference to *reservoirs* is unhelpful in understanding resistance gene flow, even in cases like *E. coli,* and is an impediment to ultimately managing the problem of clinical resistance. When we observe identical genetic elements residing in two different vectors or species, we can only say that there has been a lateral transfer event. However, we cannot reliably ascribe the direction of that transfer, nor establish that the transfer occurred directly between the two locations currently occupied by that particular genetic element. Despite this fundamental problem, an implicit assumption of direction is contained in the 'reservoir' hypothesis of antibiotic resistance (Salyers & Shoemaker, 2006). The term 'reservoir' suggests a pool of genes that flows downwards, usually into human beings and their pathogens, and this is probably too simplistic.

In contrast, we believe that most and possibly all environments/organisms act as conduits for resistance gene flow. Some organisms like *E. coli* play a particularly important role, but all bacteria may have a role to play. Global gene flow acts in multiple directions, allowing the introduction of new resistance genes into human pathogens and shuttling known, clinically important resistance genes back into the broader bacterial population for subsequent cycling into other clinical contexts (Fig. 3). The ability of organisms like *E. coli* to facilitate resistance gene flow has other possible ramifications for understanding the epidemiology of gene transfer that are not adequately conveyed by concepts of reservoirs. An alternative useful view is offered by source–sink modelling. Under this model, sinks – in this case clinical pathogens – acquire DNA from sources – the broader environment. The model is constructive because it does not preclude two-way flow, and experimental data suggest that immigration into the sink can result in faster rates of adaptation, which, in a clinical context, translates into faster adaptation to antibiotic resistance (Pulliam, 1988; Sokurenko *et al.*, 2006; Perron *et al.*, 2007). The concept is consistent with conduits because source–sink modelling provides insights into the primary direction of flow and how the process started, whereas conduits imply a mechanism of transfer. Generally, these types of models reinforce observations that resistance genes (Knapp *et al.*, 2010) and the elements that mobilize them tend to increase in abundance over time even in the absence of selection for all of their components.

The scale and evolutionary history of LGT makes it inevitable that practically any gene can find its way into any bacterial cell. The key to understanding this ongoing process is to quantify both the opportunity for lateral transfer and the strength of selection for particular transferred genes and the phenotypes they confer (Martinez *et al.*, 2007). The

Table 2. Reports of class 1 and class 2 integrons in domesticated animals and food animals, 2008–2010*

Country	Animal host	Bacterial host	*intI1*	Cassette array(s)†	*intI2*	Cassette array(s)†	Reference/s
Australia	Cattle	*Enterobacteriaceae*	+	*aadA1*, *aadA2*, *catB8-aadA1*, *cmlA5-bla*$_{OXA-10}$*-aadA1*, *dfrA1-aadA1*, *dfrA17-aadA5*, *dfrA12-orf-aadA2* *dfrA7*, *dfr2d-catB3-aadA1*, *dfrV*, *sat-orf*	+	*dfrA1-sat2-aadA1*, *estX-sat2-aadA1*, *sat2-aadA1*	Barlow *et al*. (2008, 2009)
	Pig	*S. enterica* serovars	+	*dfrA12-orfF-aadA2*, *δorfF-aadA2*	–	–	Evershed *et al*. (2009)
Brazil	Pig	*S. enterica* Bredeney	+	*dfrA12-bla*$_{OXA-129}$*-aadA1*	+	*dfrA1-sat1-aadA1*	Michael *et al*. (2008)
Canada	Chicken	*E. coli*	+	*aadA1*, *dhfrI*	–		Bonnet *et al*. (2009)
Chile	Pig	*Salmonella* spp.	+	*aadA1*	+	*dfrA1-sat1-aadA1*	San Martin *et al*. (2008)
	Chicken	*E. coli*	+	*aadA1*, *dfr-A1-aadA1*	+	*dfrA1-sat1-aadA1*, *estX-sat2-aadA1*	Lapierre *et al*. (2008)
	Pig		+	*aadA1*, *dfrA1-aadA1*	+	*dfrA1-aadA1*, *dfrA1-sat1-aadA1*, *estX-sat2-aadA1*	
Czech Republic	Cattle	*E. coli*	+	*aadA1*, *dhfr1-aadA1*, *dhfr17-aadA5*	ND		Dolejska *et al*. (2008)
	Pig	*E. coli*	+	*aadA1*, *aadA2*, *bla*$_{OXA}$*-aadA1*, *dhfr1-aadA1*, *dhfr12-aadA2*, *estX-aadA1*, *estX-aadA2*	+	*estX-sat-aadA1*, *sat-aadA1*	Literak *et al*. (2009)
Denmark	Chicken	*E. coli*	+	ND	ND		Trobos *et al*. (2008)
Egypt	Cattle	*E. coli*	+	*aac(3)-Id-aadA7* *aadA1*, *aadA23*, *dfrA1-aadA1*, *dfrA12-orf-aadA2*, *dfrA15*, *dfrA17-aadA5*	+	*dfrA1-sat2*, *dfrA1-sat2-aadA1*	Ahmed *et al*. (2009d)
	Cattle	*S. enterica* serovars	+	*aadA1*, *dfrA1-aadA1*, *aadA2*, *dfrA15*, *dfrA17-aadA5*	+	*dfrA1-sat2-aadA1*	Ahmed *et al*. (2009c)
France	Cattle	*S. enterica* Typhimurium	+	*bla*$_{PSE-1}$, *aadA2*	ND		Targant *et al*. (2010)
Germany	Cat	*E. coli*	+	*aadA1*, *dfrA1-aadA1*, *dfrA12-orfF-aadA2*	+	*dfrA1-sat2-aadA1*	Kadlec & Schwarz (2008)
	Dog		+	*aadA1*, *dfrA1-aadA1*, *dfrA1-catB3-aadA4*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5*	+	*dfrA1-sat2-aadA1*, *estX-sat2-aadA1*	
	Horse		+	*aadA1*, *dfrA1-aadA1*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5*	+	*dfrA1-sat2-aadA1*	
	Pig		+	*aadA1*, *aadB-aadA1*, *dfrA1-aadA1*, *dfrA12-orfF-aadA2*, *dfrA14-aadA6*, *dfrA17-aadA5*	+	*dfrA1-sat2-aadA1*, *estX-sat2-aadA1*	
	Cat	*Salmonella* serovars	+	*aadB*, *bla*$_{PSE-1}$	–		Rodriguez *et al*. (2009)
	Chicken		+	*aadA1*, *dfrA1-aadA1*	+	*dfrA1-sat2-aadA1*	
	Horse		+	*aadB*, *bla*$_{PSE-1}$	–		
	Pig		+	*dfrA1-aadA1*	–		
Greece	Chicken	*E. coli*	+	*aacA4-catB3-dfrA1* *aadA1*, *aadA1-dfrA1* *aadA2-orfF-dfrA12*, *aadA5-dfrA17*, *sat*	ND		Vasilakopoulou *et al*. (2009)
Hong Kong	Cattle	*E. coli*	+	*dfrA17-aadA5*	–		Ho *et al*. (2009)
	Chicken		+	*dfrA1-aadA1*	–		
	Pig		+	*aadA23*, *arr3*, *dfrA1-aadA1*, *dfrA17-aadA5*	–		
Hungary	Chicken	*S. infantis*	+	ND	ND		Nogrady *et al*. (2008)

Table 2. Continued.

Country	Animal host	Bacterial host	*intI1*	Cassette array(s)†	*intI2*	Cassette array(s)†	Reference/s
Ireland	Cattle	*E. coli*	+	*aadA1*, *dfr1-aadA1*	ND		Ekkapobyotin *et al*. (2008)
	Chicken	*S. enterica* Kentucky	+	*aadB*, *sat*	ND		Boyle *et al*. (2010)
Italy	Cattle	*E. coli*	+	ND	ND		Alessiani *et al*. (2009)
	Chicken		+	ND	ND		
Japan	Cattle	*S. enterica* Typhimurium	+	*aadA1*, *aadA2*, *aadA2-bla*$_{PSE-1}$, *bla*$_{PSE-1}$	–		Ahmed *et al*. (2009c)
	Chicken	*E. coli*	+	*aadA1*, *dfrA1-aadA1*, *dfrA1-orf*, *dfrA7*	+	*dfrA1-sat2-aadA1*	Ahmed *et al*. (2009b)
	Chicken	*Salmonella* serovars	+	*aadA1*, *aadB-catB3*	+	*estX-sat2-aadA1*	Ahmed *et al*. (2009a)
	Chicken	*Salmonella infantis*	+	*aadA1*, *aadA2*, *dfrA5*	ND		Shahada *et al*. (2010)
	Pig	*S. enterica* Typhimurium	+	*aadA1*, *aadA2*, *dhfrXII-orfF-aadA2*	–		Ahmed *et al*. (2009a), Futagawa-Saito *et al*. (2010)
	Horse	*S. enterica* Typhimurium	+	ND	ND		Niwa *et al*. (2009)
Korea	Pig	*S. enterica* Typhimurium	+	*aadA1*, *dhfrXII-orfF-aadA2*	–		Rayamajhi *et al*. (2008)
Lithuania	Cattle	*E. coli*	+	*aadA1*, *dfrA1*, *dfrA1-aadA1*, *dfrA14*, *dfrA14-aadA6*, *dfrA17*, *dfrA17-aadA5*, *estX-aadA1*	+	*dfrA1*, *dfrA1-sat-aadA1*	Povilonis *et al*. (2010), Seputiene *et al*. (2010)
	Chicken		+	*aacA4-catB3-dfrA1-orfX*, *aadA1*, *dfrA1-aadA1*, *dfrA5*, *dfrA12-orfF-aadA2*, *dfrA14*	+	*dfrA1*, *dfrA1-sat*, *dfrA1-sat-aadA1*	
	Pig		+	*aadA1*, *dfrA1*, *dfrA1-aadA1*, *dfrA12*, *estX-aadA1*	+	*dfrA1*, *dfrA1-sat-aadA1*, *estX-sat2-aadA1*	
	Chicken	*S. enterica*	+	*aadA2*, *dfrA1-aadA1*	–		Povilonis *et al*. (2010)
	Pig		+	*aadA1*, *aadA7-aadA7*, *estX-aadA1*, *dfrA12-orfF-aadA2*	–		
Mexico	Cattle	*S. enterica* Typhimurium	+	*aadA1*, *dfrA17-aadA5*, *dfrA12-orfF-aadA2*	ND		Wiesner *et al*. (2009)
	Chicken		+	*dfrA17-aadA5*, *dfrA12-orfF-aadA2*	ND		
	Pig		+	*aadA1*, *dfrA17-aadA5*, *dfrA12-orfF-aadA2*	ND		
Portugal	Chicken	*Enterobacteriaceae*	+	*aadA1*, *aadA1a*, *aadA2*, *blaP1*, *dfrA1-aadA1*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5*, *estX-aadA1*	+	*dfrA1-sat2-aadA1-orfX*, *estX-sat2-aadA1-orfX*	Machado *et al*. (2008)
	Pig		+	*aadA1*, *aadA13-estX*, *dfrA1-aadA1*, *dfrA14-aadA1-catB2*, *dfrA17-aadA5*, *estX-aadA1*	+	*aadA1*, *dfrA1-sat2-aadA1-orfX*, *estX-sat2-aadA1-orfX*	
China	Chicken	*E. coli*	+	*aadA1*, *aadA22*, *aadB-orf1-cmlA*, *arr3*, *arr3-dfr16*, *dfrA1-aadA1*, *dfrA1-orfC*, *dfrA12-aadA2*, *dfr17-aadA5*, *dhfrXII-orfF-aadA2*, *dfr1-aadA1*, *yheSΔ-yheR-kefBA*¨	+	*dfrA1-sat1-aadA1*, *dfrA1-sat1-aadA1-orfX*	Zhang *et al*. (2009c), Li *et al*. (2010b), Lu *et al*. (2010)
	Cattle	*Arcanobacterium pyogenes*	+	*aadA1-aadB-cmlA6*, *aadA5*, *aadA24-orf1*	–		Liu *et al*. (2009b)
	Cattle	*E. coli*	+	*yheSΔ-yheR-kefBA*¨	+		Song *et al*. (2010)
	Pig	*E. coli*	+	*aadA1*, *aadA2*, *aadA23B*, *blaP1a-aadA2-ereA*, *dfrA1-aacA4-catB3*, *dfrA1-aadA1*, *dfrA1-orfC*,	+	*dfrA1-sat1-aadA1*	Zhang *et al*. (2009c), Lu *et al*. (2010)

Table 2. Continued.

Country	Animal host	Bacterial host	*intI1*	Cassette array(s)†	*intI2*	Cassette array(s)†	Reference/s
				dfrA12-aadA2, *dfr17-aadA5*, *dhfrXII-orfF-aadA2*, *yheSΔ-yheR-kefBA*̈			
Saudi Arabia	Chicken	*E. coli*	+	ND	ND		Altalhi *et al*. (2009)
South Korea	Cattle	*E. coli*	+	ND	–		Unno *et al*. (2010)
	Chicken		+	ND	–		
	Duck		+	ND	–		
	Pig		+	ND	–		
Taiwan	Pig	*S. enterica* Choleraesuis	+	*aadA1*, *aadA-qacH*, *aadA22*, *dfrA1-UN*, *dfr12-orfF-aadA2*, *sat-psp-aadA2-cmlA1-*	–		Lee *et al*. (2009)
Thailand	Cattle	*Salmonella* serovars	+	*aadA2*	ND		Chuanchuen *et al*. (2010)
	Chicken, Pig	*S. enterica* serovars	+	*aadA2*, *aadA4a*, bla_{PSE-1}, *codB-dfrA12-aadA2*, *dfrA1-orfC*, *dfrA12-aadA2*, *sat-dfrA12-aadA2*, *silB*	–		Chuanchuen & Padungtod (2009)
Tunisia	Cattle	*E. coli*	+	*dfrA17-aadA5*	–		Slama *et al*. (2010)
	Chicken		+	*aadA1*, *dfrA1*, *dfrA5*, *dfrA5-ereA*, *dfrA12-orf-aadA2*, *dfrA14*, *dfrA17-aadA5*, *dfrA25 sat-psp-aadA2-cmlA1*	+	*dfrA1-satA1-aadA1-orfX*	Soufi *et al*. (2009), Slama *et al*. (2010)
	Pig		+	*dfrA1-aadA*, *dfrA17-aadA5*	–		Slama *et al*. (2010)
Turkey	Chicken	*S. enterica* Enteritidis	+	*aadA*	ND		Kalender *et al*. (2009)
UK	Pig	*E. coli*	+		–		Liu *et al*. (2009a)
USA	Cat	*E. coli*	+	*aadA1*, *aadA1-UN*	+	*dfrA1-sat1-aadA1*	Shaheen *et al*. (2010)
	Dog		+	*aadA1*, *aadA1-UN*, *dfrA1-aadA1*, *dfrA17-aadA5*, *dfrA12-aadA2*, *dfrA12-orfF-aadA2*, *aadB-aadA1d*, *aacA4-catB3-dfrA1*, *aadB-aadA1-cmlA6*	+	*dfrA1-sat1-aadA1*	Shaheen *et al*. (2010), Yang *et al*. (2010)
	Pig	*S. enterica* Heidelberg	+	*aadA1*, *aadA2*	–		Patchanee *et al*. (2008), Lynne *et al*. (2009)
	Cattle		+	*aadA1*, *aadA2*, *aadA5*, *aadA9*, *dhfr*	–		Lynne *et al*. (2009)
	Turkey		+	*aadA1*	–		
	Cattle	*Salmonella* serovars	+	ND	ND		Alam *et al*. (2009)
	Turkey	*Salmonella* serovars	+	*aadA1*, *aadA2*	ND		Nde & Logue (2008), Zou *et al*. (2009)
	Turkey	*S. enterica* serovars	+	*aadA*, *aadB*, and *dfr* variants	ND		Zhao *et al*. (2009)
Vietnam	Cattle	*Salmonella* serovars	+	*aadA2*	ND		Vo *et al*. (2010)
	Chicken		+	bla_{PSE-1}, *dfrA1-orfC*	ND		
	Pig		+	*aadA2*, bla_{PSE-1}	ND		

*Table is based on English-language papers published between 2008 and 2010 recovered from PubMed using the search terms integron × animal.

†Gene and cassette nomenclature is based on that used in the original papers. In some cases, gene names are at variance with standard gene naming nomenclature. A standardized nomenclature has recently been published by Partridge *et al*. (2009). Individual cassette arrays are given on separate lines.

ND, Not determined.

Table 3. Reports of class 1 and class 2 integrons in wild animals and environmental samples, 2008–2010*

Country	Animal host/environment	Bacterial host/source	*intI1*	Cassette array(s)†	*intI2*‡	Reference
Argentina	River water	*Pseudomonas* sp.	ND		+	Ramirez *et al*. (2010)
Australia	Freshwater sediment	Diverse heterotrophs	+	*aadA11*, *orf3-qacF*, novel ORFs, no cassette	ND	Rosewarne *et al*. (2010)
	Freshwater sediment	Metagenomic	+	ND	ND	Hardwick *et al*. (2008)
	Freshwater biofilm	Metagenomic	+	Diverse, novel ORFs linked to members of the *qac* gene family	–	Gillings *et al*. (2009a)
	Soil, water, biofilm		+	Diverse, novel ORFs		Gillings *et al*. (2008)
	Prawn	*Acinetobacter* sp.	+	*msrB/msrA/ctr-aadA2*	–	Gillings *et al*. (2009b)
Canada	Atlantic salmon	*Aeromonas salmonicida*	+	*aadA7*	–	McIntosh *et al*. (2008)
	Polluted estuary	Metagenomic	+	Diverse, novel ORFs	–	Koenig *et al*. (2009)
Columbia	*Paracheridon exelrodi*	*Aeromonas hydrophila*	+	Cassettes include *dfr12*, *aac61b*, *aadA1*	ND	Verner-Jeffreys *et al*. (2009)
	Corydora melanistus	*Aeromonas hydrophila*	+	Cassettes include *dfr12*, *aadA2*	ND	
	Carriage water	Metagenomic	+	Cassettes include *aadA1*, *dfrA21*, *dfrA22*, *dfrA23*, *qacE2*	ND	
Czech	Pigeon	*E. coli*	+	No cassette	+	Radimersky *et al*. (2010)
Republic	Black-headed gull	*E. coli*	+	*aadA1*, *aadA2*, bla_{OXA-1}-*aadA1*, *dhfr1*, *dhfr1-aadA1*, *dhfr1-catB3-aadA4*, *dhfr17-aadA5*	+	Dolejska *et al*. (2009)
	Pond water	*E. coli*	+	*aadA5*, *dhfr1-aadA1*, *dhfr12-aadA2*	–	
	Koi carp	*Aeromonas* spp.	+	*aadA1*, *aadA2*, *dhfr12-aadA2*	–	Cizek *et al*. (2010)
France	Estuarine water	*E. coli*	+	*aadA1*, *dfrA1-aadA1*, *dfrA5-ereA2*, *dfrA17-aadA5*	+	Laroche *et al*. (2009)
Germany	Manured soil	Metagenomic	+	*aadA1*, *aadA2*, *aadA9*, *aadA11*, *aadA13*	ND	Binh *et al*. (2009)
Guyana	Three lined pencil fish	*Aeromonas hydrophila*	+	Cassettes include *dfr12*, *orfF*	ND	Verner-Jeffreys *et al*. (2009)
	Silver hatchet	*Aeromonas hydrophila*	+	Cassettes include *aadA2*, *dfr12*	ND	
	Carriage water	Metagenomic	+	Cassettes include *aadA1*, *aadA2*, *dfrA5*, *dfrA17*, *dfrA27*, *qacE2*	ND	
India	River water	*Acinetobacter johnsonii*	+	*dfrA28-aadA1*	–	Kumar *et al*. (2010)
Italy	Herring gull	*E. coli*	+	*aadB*, *aadA1a*, *dfrA17-aadA5*, *estX-aadA1a*	ND	Gionechetti *et al*. (2008)
	Herring gull	*Proteus mirabilis*	+	*aadB-aadA2*, *aacCA5-aadA7*, *dfrA1-aadA1a*, *dfrA15*, *estX*, *estX-smr-2-aadA1a*, *orf1-cat-orf2-aadA1a*	ND	
	Urban wastewater	*Pseudomonas* spp.	+	bla_{IMP-22}-*orfXX*, bla_{IMP-22}-*orfXX-aacA4*	–	Pellegrini *et al*. (2009)
Mexico	Dust	*E. coli*	+	ND	ND	Diaz-Mejia *et al*. (2008)
	Activated sludge tank	*E. coli*	+	ND	ND	
Mozambique	Waste water	*Vibrio* spp.	+	*aadA2*	–	Taviani *et al*. (2008)
	Posttreatment water	*Vibrio cholerae*	+	*blaP1*, *dfrA15*	–	
Portugal	Buzzard	*E. coli*	+	ND	–	Radhouani *et al*. (2010)
	Yellow-legged gull	*E. coli*	+	*aadA*, *dfrA1-aadA1*, *dfrA12-orfF-aadA2*, *sat-psp-aadA2*	+	Radhouani *et al*. (2009)
	Seagull species	*E. coli*	+	bla_{OXA-1}-*aadA1*, *dfrA1-aadA1*, *sat-psp-aadA*, *sat-aadA1*	+	Poeta *et al*. (2008)
	Wild boar	*E. coli*	+	ND	+	Poeta *et al*. (2009)
China	Wastewater	Diverse species	+	*aacA4*, *aadA1*, *aadA5*, *aadB-qacH*, *aadA4a*, *aadA11b*	ND	Li *et al*. (2009a)
	Lake, river, sediment	Metagenomic	+	ND	ND	Zhang *et al*. (2009a)
	Sewage treatment	Metagenomic	+	ND	ND	
	Wastewater	*Gammaproteobacteria*	+	Cassettes include *aadA1*, *aadA2*, *aadA2a*, *dfrA1*, *dfrA12*, *qacG*	ND	Li *et al*. (2010a)
	Wastewater	*Aeromonas punctata*	+	*aacA4-qnrVC4-aacA4-catB3*	–	Xia *et al*. (2010)
Singapore	Guppy	*Aeromonas hydrophila*	+	*dfr12*	ND	Verner-Jeffreys *et al*. (2009)

Table 3. Continued.

Country	Animal host/environment	Bacterial host/source	*intI1*	Cassette array(s)†	*intI2*‡	Reference
	Harlequin rasbora	*Aeromonas hydrophila*	+	*dfrA1*	ND	
	Redwag platy	*Aeromonas punctata*	+	Cassettes include *dfr12*, *aac61b*, *aadA1*, *catB8*	ND	
	Carriage water	Metagenomic	+	Cassettes include *aadA1*, *aadA2*, *dfrA1*	ND	
Switzerland	Lake water	*Aeromonas allosaccharophila*	+	*aac61b-bla*$_{OXA-1}$*-catB3-arr3*	ND	Picao *et al*. (2008)
Tanzania	Flamingo	*E. coli*	+	*dfrA7*	+	Sato *et al*. (2009)
	Flamingo	*Salmonella arizonae*	+	*dfrA7*	−	
Turkey	River water	*E. coli*	+	Cassettes include *aadA1*, *aadA5*, *bla*$_{OXA30}$, *dfrA1*, *dfr2d*, *dfrA7*, *dfrA16*, *dfrA17*, *sat1*	+	Ozgumus *et al*. (2009)
UK	Koi carp	*Aeromonas hydrophila*	+	Cassettes include *aadA1*, *dfrA1*	ND	Verner-Jeffreys *et al*. (2009)
	Agricultural soil	Diverse species	+	ND	+	Byrne-Bailey *et al*. (2009)
USA	Catfish	*Aeromonas veronii*	+	ND	−	Nawaz *et al*. (2010)
	Catfish	*E. coli*	+	*dfrA17-aadA5*, *dfrA12-orfF-aadA2*	ND	Nawaz *et al*. (2009)
	Forest soil	Diverse species	+	No cassettes	ND	Srinivasan *et al*. (2008)
	Dairy soil	*Citrobacter* spp. others	+	*aadA2*, *dfrA12-aadA2*	ND	
	Soil, water, compost, manure	Diverse species	+	*aadA1*, *aadA7*, *aadA9*, *dfr16*	ND	Yang *et al*. (2010)
	Estuarine habitat	Metagenomic	+	Diverse, novel ORFs	ND	Wright *et al*. (2008)
	Riverine habitat	Metagenomic	+	Diverse, novel ORFs	ND	
	Sewage treatment	Metagenomic	+	ND	ND	Ghosh *et al*. (2009)
	Compost	*E. coli*	+	*aadA1*, *aadA1-dfrA1*, *aadA1-dfrA15*, *aadA2-dfrA12*	ND	Heringa *et al*. (2010)

*Table is based on English-language papers published between 2008 and 2010 recovered from PubMed using the search terms integron × environment.
†Gene and cassette nomenclature is based on that used in the original papers. In some cases, gene names are at variance with standard gene naming nomenclature. A standardized nomenclature has recently been published by Partridge *et al*. (2009). Individual cassette arrays are given on separate lines, although in some cases, full cassette arrays were not characterized, and entries list gene cassettes detected.
‡Cassette arrays for class 2 integrons were *dfrA1-sat2-aadA1* or *setX-sat-aadA1*.
ND, not determined.

specific lateral transfer events that have led to the current crisis in antibiotic resistance management have probably occurred many thousands of times in the evolutionary past, but under circumstances where they conferred no selective advantage. Under strong selection, however, antibiotic resistance genes can come to dominate a population or, with LGT, an entire community. While it is the case that the acquisition of an antibiotic resistance gene can come at a fitness cost, at least initially, compensatory mutations can arise to counteract this (Andersson, 2003). Thus, a reduction in antibiotic usage does not necessarily lead to a significant reduction in resistance gene frequency in a population.

Understanding the dissemination of resistance determinants requires the investigation of all the locations where antibiotic-resistant Bacteria might reside. There are five sources/sinks that are of particular interest: human beings, domestic animals, companion animals, wild animals and the general environment (Fig. 3). The high level of interest in the dissemination of resistance genes between these locations can be gauged by the contents of Tables 2 and 3, which deal with recent reports of integrons in animals and in the general environment. As schematically illustrated in Fig. 3, transfers of mobile elements and linked resistance genes (integrons or otherwise) can potentially occur directly between any of these sources/sinks, and it is likely that that new vectors or combinations of resistance genes arising in one host can rapidly circulate through all locations.

Of all the mobile elements in Gram-negative bacteria, class 1 integrons have been most extensively surveyed in clinical contexts. As we will discuss below, the ease with which integrons can be surveyed is itself an impediment, because it introduces a bias in focus, and is not helpful in understanding the resistance problem from the perspective of the microbial biosphere. Bearing this caveat in mind, it is still the case that there is a wealth of published evidence for the LGT of integrons, mobilized by other elements or processes, between most of the sources and sinks depicted in Fig. 3. We will give some examples of transfers of particular interest, but unfortunately cannot cover the complete literature in this review.

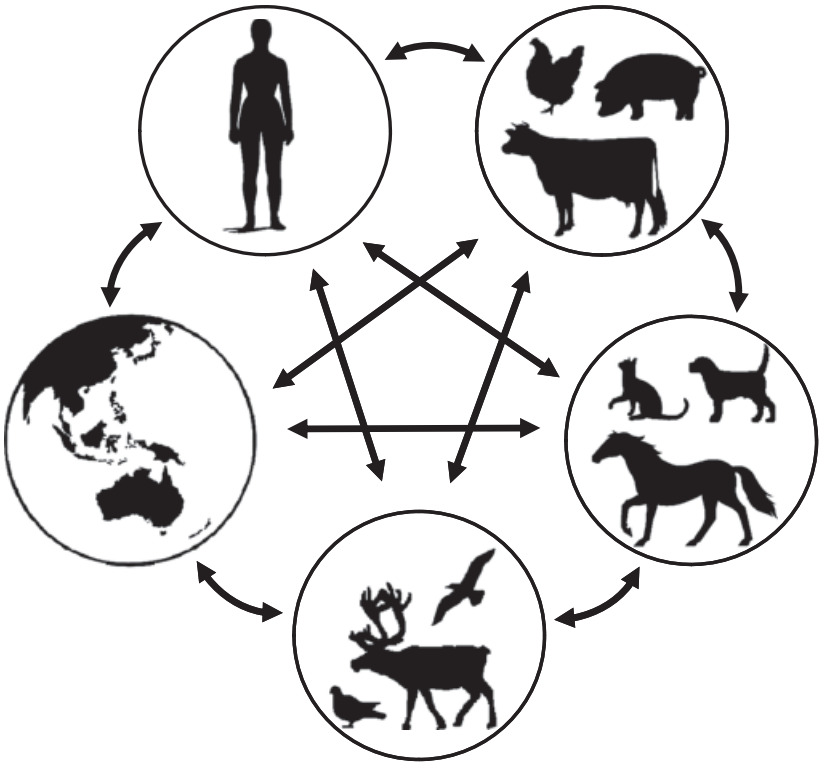

Fig. 3. Conduits of gene transfer between different bacterial species, animal hosts and the environment. Genes can potentially move directly between various host environments including humans, food animals, domestic animals, wild animals and the general environment. In the case of gene cassettes containing antibiotic resistance determinants, this lateral transfer may be mediated via transformation with free gene cassettes, integrons, transposons or plasmids. Alternately, genes can spread by conjugation between various commensals and pathogens transferred between host environments.

Transfer between humans and their domestic animals

Class 1 and class 2 integrons are widely disseminated in *E. coli* and *Salmonella* isolated from a range of companion and food animals (Table 2). These integrons are in many cases identical to those found in human commensals and pathogens, establishing that a conduit of lateral transfer exists between humans and these animals (Goldstein *et al.*, 2001; Schwarz & Chaslus-Dancla, 2001; Schwarz *et al.*, 2001; Antunes *et al.*, 2006; Hsu *et al.*, 2006; van Essen-Zandbergen *et al.*, 2007). The presence of identical integrons and cassette arrays in various *Salmonella* serovars and *E. coli* strains demonstrates that lateral transfer occurs within and between these species, even when residing in different hosts (Box *et al.*, 2005; Singh *et al.*, 2005; Hammerum *et al.*, 2006; Ajiboye *et al.*, 2009). Integrons have also spread from the terrestrial environment into *E. coli* and *Aeromonas* strains associated with aquaculture (Cabello, 2006; McIntosh *et al.*, 2008; Nawaz *et al.*, 2009, 2010). Because the prevalence of integrons in all animals increases with vicinity to humans, it has been suggested that the presence of integrons in animals is due to transmission from humans rather than the reverse (Skurnik *et al.*, 2006).

Transfer between humans/domestic animals and wild animals

There are difficulties in establishing the directionality of transfer of bacteria or genes. Nevertheless, a good case can be made in some instances (Table 3). A number of studies have suggested that wild birds such as gulls and pigeons can acquire integrons and antibiotic resistance genes by feeding on waste or from contaminated waters (Gionechetti *et al.*, 2008; Poeta *et al.*, 2008; Bonnedahl *et al.*, 2009; Dolejska *et al.*, 2009; Radimersky *et al.*, 2010). Anthropogenic transfer of integrons and resistance genes to wild boars and buzzards has also been suggested (Poeta *et al.*, 2009; Literak *et al.*, 2010; Radhouani *et al.*, 2010). It seems very likely that integrons in wild animals, zoo animals and ornamental fish (Ahmed *et al.*, 2007; Sato *et al.*, 2009; Verner-Jeffreys *et al.*, 2009) (Table 3) have their origins in humans or their domesticated animals. There also appears to be transfer of enterobacteria and resistance determinants between humans and chimpanzees in Uganda (Goldberg *et al.*, 2007).

Transfer between humans/domestic animals and the more general environment

Integrons from animal husbandry operations make their way into the general environment via the spread of manure (Agerso & Sandvang, 2005; Binh *et al.*, 2009; Byrne-Bailey *et al.*, 2009) and in compost (Heringa *et al.*, 2010; Yang *et al.*, 2010) (Table 3). Human waste streams disseminate integrons and resistance genes, initially to wastewater treatment plants (Schluter *et al.*, 2007; Ghosh *et al.*, 2009; Zhang *et al.*, 2009b), but also more generally into rivers and estuaries (Laroche *et al.*, 2009). *Escherichia coli* strains containing

plasmids and multidrug resistance have even made their way into remote areas, including isolated communities in the Peruvian Amazonas and birds in the Arctic (Sjolund *et al.*, 2008; Bartoloni *et al.*, 2009).

Potential for transfer back into human populations

When antibiotic resistance genes and vectors are spread from human-dominated ecosystems, they can penetrate new bacterial hosts. Transfer of integron-mediated antibiotic resistance between *E. coli* strains has been demonstrated in bovine faeces and in storm water (Nagachinta & Chen, 2008). Similarly, a class 1 integron carrying a novel bla_{IMP} has moved from *Pseudomonas aeruginosa* in a nosocomial environment into *P. fluorescens* in wastewater (Pellegrini *et al.*, 2009). Such activity has the potential to generate novel opportunistic pathogens. Antibiotic resistance integrons may also interact with diverse mobile elements in the environment and acquire new resistance and pathogenicity determinants. Novel integron gene cassettes conferring trimethoprim and quinolone resistance have recently been recovered from environmental *Acinetobacter* and *Aeromonas* isolates, respectively (Kumar *et al.*, 2010; Xia *et al.*, 2010). Clinical class 1 integrons have made their way into the commensal bacteria of wild animals, where they continue to acquire novel cassettes. A class 1 integron has been described in *E. coli* from a wild reindeer that carried an *ant(3″)-Ia* resistance cassette, and had also acquired a gene cassette with homology to a cassette of unknown function described from *Xanthomonas* (Sunde, 2005).

Does human use of antimicrobial agents change the tempo of lateral transfer?

It has been argued that humans are the 'World's greatest evolutionary force' (Palumbi, 2001), a recognition of our ability to engineer the biosphere. One particularly apt example of this concept is our impact on the accelerated evolution of antibiotic resistance. The use of antibiotics may have changed the dynamics of bacterial evolution by increasing the basal rate of mutation, enhancing LGT and promoting the generation of novel DNA elements. We know that antimicrobial compounds induce stress in bacterial cells, leading to changes in transcriptomic profiles (Davies *et al.*, 2006) and other mechanisms that increase evolvability (Baquero, 2009). For instance, the evolution of resistance in *E. coli* is accelerated by exposure to multiple antibiotics (Hegreness *et al.*, 2008).

It is likely that the ecology of human dominated ecosystems helps to stimulate the generation of novel genetic elements. Exposure of cells to various antibiotics induces an SOS response that has widespread effects on the bacterial genome (Miller *et al.*, 2004; Aertsen & Michiels, 2006). These responses include promoting lateral transfer of antibiotic resistance genes (Beaber *et al.*, 2004) and increasing integron recombination events (Guerin *et al.*, 2009). Consequently, the release of antibiotics in human waste streams is likely to have an impact on LGT and on the activity and complexity of large gene cassette arrays carried by the diverse chromosomal integrons found in environmental samples.

Waste streams from human-dominated ecosystems simultaneously release resistance determinants, their DNA vectors and the antimicrobial agents that select for them (Baquero *et al.*, 2008; Martinez, 2009). Wastewater brings together diverse cells, plasmids, integrons and resistance genes, creating a hotspot for interaction between these elements in an environment that contains subinhibitory concentrations of the selective agents to which they exhibit resistance (Schluter *et al.*, 2007, 2008; Moura *et al.*, 2010). Such environments allow complex mosaics of subelements to be built up (Toussaint & Merlin, 2002; Norman *et al.*, 2009), and because these complex DNA elements share homologous regions, recombination is enhanced, thus promoting still further diversity (Garriss *et al.*, 2009).

It is clear that the fixation of antimicrobial resistance genes in pathogens and commensals is driven by the selection imposed by antibiotics. The transfer of such genes has been occurring for millennia, but not necessarily under conditions where such transfers conferred any advantage to the recipient cell. The propensity for such LGTs should be balanced by two opposing selective forces: the potential advantage accrued through the acquisition of foreign genes, balanced against the deleterious effects of invasion by transposons, bacteriophage and other detrimental DNA elements (Fig. 4).

It therefore seems reasonable to suggest that porosity to lateral transfer is under balancing selection, and that different species, and even cells within species, fall along a gradient of porosity. The widespread human use of antibiotics and their distribution via human waste streams (Baquero *et al.*, 2008; Martinez, 2009) may have altered the strength of this balancing selection, such that cells and species with a greater porosity to lateral transfer have an inherent advantage because some members of their lineage will acquire and express resistance genes. Hence, it is highly probable that the general tempo of lateral transfer has actually increased due to selection on cells with inherently higher rates of lateral transfer (Fig. 4).

The recent discovery of the CRISPR system provides a potential mechanism for restricting the uptake of foreign DNA. Changes to this system may have the result of modulating the rate of uptake of DNA mobilized by LGT. The clustered regularly interspaced short palindromic repeat (CRISPR) system is a bacterial form of acquired immunity (van der Oost *et al.*, 2009). The CRISPR system is complex

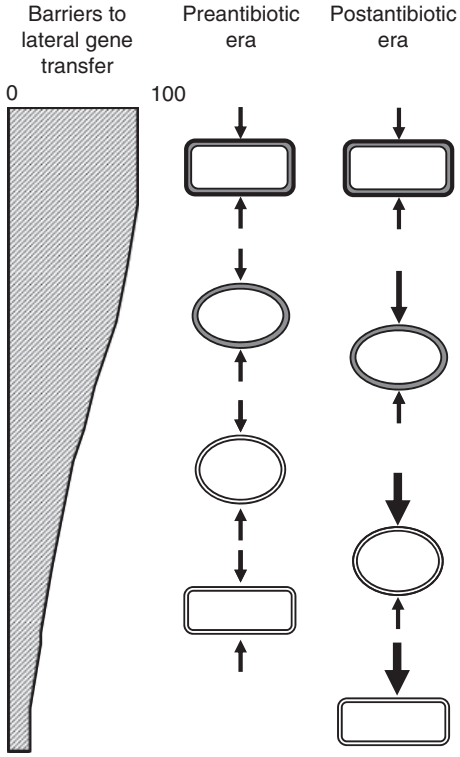

Fig. 4. Barriers to lateral gene transfer. For any species, the barriers to lateral gene transfer are set by two opposing and balanced selective forces: the ability to resist infection by bacteriophage and/or barriers to transposons (upward arrows) and the advantage conferred by the ability to acquire new phenotypes (downward arrows). The widespread dissemination of antibiotics may have altered this equilibrium, selecting for increased lateral transfer capability.

and remarkably sophisticated, but the key component providing the acquired immunity is the presence of small RNA guides that target specific DNA sequences that act to prevent the incorporation of incoming DNA that possess complementary sequences. The system is analogous, but unrelated to interfering RNA found in eukaryotes. The extent to which a bacterial cell can limit LGT is determined by the number and type of RNA guides, which in turn varies between individual cells (Marraffini & Sontheimer, 2008). Genomic analysis would suggest that CRISPR systems are common among the prokaryotes (van der Oost *et al.*, 2009) and that they may influence the evolution of pathogenesis (Marraffini, 2010). The CRISPR system is likely to be important in influencing cell fitness because it has been argued that it may help in providing a balance between the potential positive vs. the negative impacts of acquiring DNA by LGT (van der Oost *et al.*, 2009). Coevolution of the system with its host should occur such that in environments where acquiring DNA may be detrimental, an increase in the CRISPR content would occur and a decrease would occur where 'foreign' DNA may be advantageous (Vale & Little, 2010). This has direct relevance for the spread of antibiotic resistance because the global use of antibiotics could see a substantial decrease in the CRISPR content in bacterial populations, leading to a general increase in the rate of mobilization of DNA by LGT as measured by successful transfer events. In support of this, an inverse correlation between CRISPR content and the extent of multidrug resistance has been reported recently (Palmer & Gilmore, 2010).

The agents of gene capture and spread

All of the mobilizing elements associated with the movement of resistance genes in pathogens long predate the antibiotic era. Analysis of at least one extensive strain collection dating from the early 20th century demonstrated that plasmids were a common feature of strains of the *Enterobacteriaceae* (Hughes & Datta, 1983; Jones & Stanley, 1992). Similarly, transposons were a common feature of soil dwelling bacteria in the preindustrial era (Kholodii *et al.*, 2003; Mindlin *et al.*, 2005). Integrons, based on their phylogenetic history, have been features of bacterial chromosomes for at least several hundred million years (Mazel, 2006; Boucher *et al.*, 2007). Where they can be directly examined, mobile elements from before the antibiotic era are clearly related to contemporary elements that carry resistance genes. Thus, preantibiotic era plasmids belong to the same incompatibility groups as those seen today (Hughes & Datta, 1983) and bacteria from ancient permafrost possess mercury resistance transposons related to those found in pathogens (Mindlin *et al.*, 2001, 2005). While the evidence is more circumstantial, it is also likely that the class 1 integron, responsible for spreading resistance genes, was quite broadly distributed in the *Proteobacteria* before the antibiotic era (Stokes *et al.*, 2006; Gillings *et al.*, 2008). What does distinguish these mobile DNA elements in early isolates from those in contemporary pathogens is that the former are rarely found in association with antibiotic resistance genes, whereas the latter are substantial carriers of these genes. Secondly, in contemporary bacteria, a high degree of clustering is observed such that multidrug resistance regions are commonly mosaics made up of many of the mobile genetic elements described. This cooperation between disparate mechanisms of LGT has considerably facilitated the global spread of resistance genes (Walsh, 2006) and is contributing to the evolution of multidrug-resistant pathogens with enhanced virulence, via the accumulation of disparate virulence factors in pathogenicity islands (Juhas *et al.*, 2009) and virulence plasmids (Villa & Carattoli, 2005).

With time and ongoing selection, the trend is towards increasing complexity of multidrug resistance elements. This increase in complexity is driven by combinatorial exchanges between existing elements, recruitment of new

elements (Walsh, 2006; Garriss *et al.*, 2009) and by coselection for genes that confer resistance to environmental compounds and pollutants (Baker-Austin *et al.*, 2006).

Plasmids

The central role of plasmids in contributing to LGT is not disputed. It was the discovery of these genetic agents that led to a paradigm shift where bacterial evolution was viewed as more than spontaneous mutation and binary fission of haploid cells (Lederberg & Tatum, 1946). It was then described by Salvador Luria as '... among the most fundamental advances in the whole history of bacteriological science' (Luria, 1947). Given subsequent developments, including the key role of plasmids in making the gene cloning revolution possible, the statement is still true. The central role of plasmids is inferred in Table 1, where it can be seen that at least some plasmids are autonomous, both with respect to their physical movement (via conjugation) and through their autonomous replication. Notwithstanding these basics, plasmids are extraordinarily versatile in that their size and (commonly) circular form means that they are readily mobilizable by transformation, a factor that may be important in soil-dwelling organisms (Sikorski *et al.*, 2002) and represents an obvious mechanism for the spread of nonconjugative plasmids.

Once direct evidence for the existence of extrachromosomal DNA in bacteria was established, the early years of plasmid biology were dominated by the characterization of their basic properties including size, the fundamentals of incompatibility, entry exclusion and the replication and transfer genes they carried (Novick, 1969). The link between plasmids and resistance genes was noted very early and resulted in *P. aeruginosa* emerging as a major focus of genetic study in the Gram negatives (Holloway, 1969). The reasons for this included the fact that it is a significant opportunistic pathogen and a common cause of nosocomial infections. In a plasmid context, *P. aeruginosa* is a source of a number of resistance (or RP) factors and many of these were unusual at the time in that they were able to easily cross species boundaries (Sykes & Richmond, 1970; Grinsted *et al.*, 1972). With the characterization of these resistance factors, it was realized that the emergence of multidrug resistance was a result of the comobilization of several genes and that this was now a major clinical problem, not just a rare inconvenience involving the transfer of resistance to a specific single drug (Anonymous, 1974). This same period also saw the onset of the first resistance epidemiology studies and the establishment of the link between the clinical use of antibiotics and an increase in resistance carriage (James *et al.*, 1975; Krcmery *et al.*, 1975). Ironically, it was just at this time that the potential of plasmids to play a role in adaptation to the clinical application of antibiotics was becoming obvious. Also, at this time, more early data hinted at the presence of other mechanisms of resistance gene mobilization. Specifically, it was found that R plasmids could be targets for translocating pieces of DNA that carried resistance genes and that the process by which this occurred was independent of homologous recombination pathways (Bennett & Richmond, 1976). Thus, the fundamental advances first articulated by Salvador Luria 30 years previously were clearly all still to be unravelled. The systems biology approaches available in contemporary times to analyse mobile DNA, although more sophisticated, make it clear that the role played by plasmids in particular to bacterial adaptation is a long way from being fully understood (Johnson & Nolan, 2009; Halary *et al.*, 2010; Smillie *et al.*, 2010).

Transposons

Why are some mobile elements more commonly associated with antibiotic resistance genes in pathogens than others? There may be a number of reasons for this outcome, including an element of chance. Chance may play a role, since the first capture event(s) of resistance gene(s) in a particular element remove the selective advantage conferred by infiltration of other elements carrying the same or similar genes. Alternatively, some groups of mobile elements may have been 'primed' for infiltration into pathogens. This is notably the case for mercury-resistant transposons existing before the antibiotic era (Fig. 5). The redox potential of mercury can vary, but only the oxidized state is toxic (Foster, 1987). The bacterial inactivation of mercury is via a reduction reaction. The proteins that detoxify mercury therefore probably first arose when the biosphere became oxygenated (Barkay *et al.*, 2010) and mercury resistance transposons have been a feature of soil-dwelling bacteria for a very long time (Mindlin *et al.*, 2001, 2005). Many of these are very closely related to multidrug resistance transposons in contemporary pathogenic isolates (Kholodii *et al.*, 2003). Having begun to capture resistance genes, particular types of these transposons demonstrated very rapid rates of evolution, driven by selection pressure in the antibiotic era. The best examples of this phenomenon are derivatives of the Tn*21* family (Liebert *et al.*, 1999). Before human impacts, mercury was present in some environments at low levels and it likely that bacteria play a role in the global cycling of this element (Baldi, 1997). Even in pristine and/or preindustrial era soils, resident bacteria can possess highly evolved and regulated operons for the chemical transformation of mercury (Barkay *et al.*, 2003; Barkay & Wagner-Dobler, 2005). In environments where anthropogenic disturbance has seen the introduction of elevated levels of mercury, corresponding enrichment for resistant bacteria has occurred (Barkay & Pritchard, 1988; Sprocati *et al.*, 2006). This enrichment was probably occurring before the antibiotic era both in the general environment

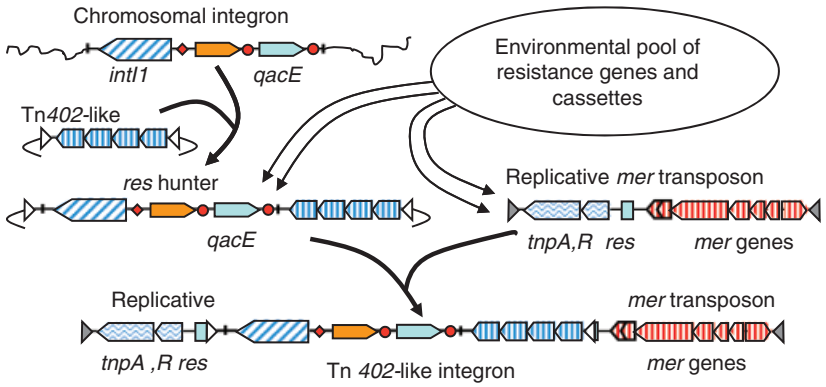

Fig. 5. Coselection and recruitment of transposons and class 1 integrons into pathogens. The schematic represents a model describing the order of events leading to complex and highly mobile multiresistance regions in contemporary Gram-negative pathogens. The use of disinfectants led to the linking of *qac* genes to class 1 integrons before the antibiotic era. This structure then linked to a Tn*402*-like transposition module and became mobile. At about the same time, with the onset of extreme selection pressure via the use of antibiotics, antibiotic resistance genes began to be recruited into this structure and its descendants. In parallel, the presence of mercury in the environment – both natural and human induced – led to the enrichment for mercury-resistant transposons. These also began to independently recruit resistance genes from the onset of the antibiotic era. At the same time and subsequently, the *res* targeting mechanism associated with Tn*402*-like class 1 integrons made the linking of a broad range of transposition modules to site-specific recombination functions inevitable.

and through the use of topical disinfectants that included mercury. The mining industry has also contributed to the increased resistance in the general environment throughout the industrial era (Ball *et al.*, 2007).

Another source of relatively high concentrations of mercury is amalgam dental fillings. From an ecological perspective, this is potentially an important one because it is an environment at the interface of commensal and human pathogenic bacteria. Some early data suggested that mercury released from such fillings can promote an increase in both mercury and antibiotic resistance (Summers *et al.*, 1993). The idea was considered controversial, at least by the dental community (Shearer, 1993), and more recently, some studies adopt a more equivocal stand on the notion that a strong link exists (Roberts *et al.*, 2008b). However, similar to trying to identify specific reservoirs of resistance, these studies probably serve to underscore the difficulty of identifying single specific causes for a problem that is multifactorial in nature. Thus, dental amalgams are possibly one further step in the enrichment for antibiotic resistance, but not the single cause of that selection. Whatever the main driving forces, it is clear that antibiotic resistance genes began to appear on mercury resistant transposons and plasmids soon after the clinical introduction of antibiotics (Smith, 1967) and that co-selection is driving the increasing linkage of resistance genes to mercury resistance transposons.

Whatever the cause, it is clear that there is a strong link between antibiotic resistance and mercury resistance. While there are many genetic contexts in which antibiotic resistance and mercury resistance genes are found together, probably the most prevalent linkage is that between class 1 integrons and specific families of mercury resistance transposons. Of the latter, the most prevalent includes the Tn*21* transposon family (Liebert *et al.*, 1999). The success of the class 1 integron/Tn*21* combination can be partly explained by the *res* hunting ability of the Tn*402* transposition system and the fact that Tn*21* and its relatives were already widespread in environmental bacteria before the onset of the antibiotic era (Mindlin *et al.*, 2001, 2005; Kholodii *et al.*, 2003). In contemporary times, this linkage between antibiotic resistance and mercury resistance may see coselection work 'both ways' to reinforce the link. That is, selection for antibiotic resistance may facilitate an increase in mercury resistance genes in bacteria and selection for mercury resistance via the environmental presence of mercury (whether natural or human induced) may be facilitating the retention of antibiotic resistance genes (Summers *et al.*, 1993; Skurnik *et al.*, 2010).

Integrons

The class 1 integrons provide an unrelated, but parallel example of selection priming. Integrons have been present in the Proteobacteria for a very long time (Mazel, 2006; Boucher *et al.*, 2007). The defining feature of these elements is the presence of a site-specific recombination system capable of capturing individual genes when part of mobile gene cassettes (Martinez & de la Cruz, 1988; Stokes & Hall, 1989; Demarre *et al.*, 2007). Over 100 classes of this element have been identified based on differences in the amino acid sequence of the site-specific recombinase protein, IntI. Although there may be site selection differences and modulation of the recombination reaction based on environmental parameters, it is nonetheless the case that

members of different integron classes appear to operate by essentially the same biochemical process (Biskri *et al*., 2005; Guerin *et al*., 2009; Shearer & Summers, 2009). Why then are the class 1 integrons almost exclusively responsible for disseminating resistance genes by site-specific recombination in Gram-negative pathogens, and why are they so abundant in these same organisms (Partridge *et al*., 2009)? Integrons are generally located in bacterial chromosomes and most are not readily mobile, although they do show evidence having moved by LGT over evolutionary time periods (Boucher *et al*., 2007). The class 1 integrons from multi-drug-resistant pathogens, however, are highly mobile and are embedded in a plethora of mobile elements, including plasmids and transposons or, more frequently, both. In particular, the 'clinical' type of class 1 integron is found in association with the remnants of a transposon, the functional exemplar of which is Tn*402*, also known as Tn*5090* (Shapiro & Sporn, 1977; Radstrom *et al*., 1994). When first named, the definition of integrons was based on both the function (the components of the site-specific recombination system) and the structural features of the clinical type of class 1 integrons that included all of the sequence common to this type when different examples were compared. This structural definition therefore additionally encompassed sequences between the Tn*402*-like inverted repeats designated IRi and IRt (Stokes & Hall, 1989; Partridge *et al*., 2001), reflecting the apparent universal association of class 1 integrons with Tn*402*-like transposition functions. In these same class 1 integrons, part of the transposition module had been lost and replaced with a 3′-conserved segment (3′-CS) (Stokes & Hall, 1989). Despite the fact that these integrons were defective transposons, transposition could still occur if deleted functions were provided *in trans* (Brown *et al*., 1996). This association with a transposon is regarded as a key step in promoting the LGT of this integron class in pathogens (Liebert *et al*., 1999).

Tn*402* and relatives are examples of transposons that are *res* hunters, meaning that the transposition event targets the resolution regions important in the replication and/or the mobility of many plasmids and transposons (Kholodii *et al*., 1995; Petrovski & Stanisich, 2010). This ability to target other mobile elements was another key factor in the spread of class-1-associated antibiotic resistance genes from the earliest days of the antibiotic era. Thus, numerous examples are known of plasmids originally isolated in the 1950s and 1960s that carry resistance genes as a consequence of either the direct insertion of a class 1 integron into a plasmid *res* site (Brown & Willetts, 1981; Ward & Grinsted, 1982; Swedberg & Skold, 1983; Hall & Vockler, 1987) or via the acquisition of a transposon such as Tn*21* (Swedberg & Skold, 1983; Hall & Vockler, 1987) that itself had captured a class 1 integron.

The *res* hunter family of transposons is commonly associated with mercury resistance, although in the Tn*402*-like integron version the integron module has replaced the *mer* module (Kholodii *et al*., 1995). In most clinical class 1 integrons, the *tni* module has undergone one or more deletions after the acquisition of the 3′-CS, which includes a sulphonamide resistance determinant. Class 1 integrons/transposons with a complete transposition module and no 3′-CS are known, although they are uncommon, and so the acquisition of the 3′-CS is generally presumed to have occurred soon after the clinical application of antibiotics (Brown *et al*., 1996). Very recently, however, this has been called into question with the discovery from permafrost dating back at least 15 000 years of a *Pseudomonas* sp. that possesses a class 1 integron with all the features seen in clinical isolates including a known resistance gene cassette and a 3′-CS (Petrova *et al*., 2011). This observation, if correct, does not necessarily change the order of steps involved in the evolution of the clinical type of class 1 integron, but would obviously impact on the timing. In our view, this observation is so radical as to require interpretation with caution. In particular, it needs to be established that contamination with contemporary isolates has not occurred and additional independent isolates need to be found.

While the defective integron/transposon version is undoubtedly the most common type of class 1 integrons in clinical isolates, the functional ancestor may also be more frequent than realized because the most common form of PCR screening for class 1 integrons is based on a primer that targets the 3′-CS (Levesque *et al*., 1995). Consequently, testing for other variants, most notably those with a complete transposition module (Post *et al*., 2007), would be highly desirable. Putting aside differences in the inserted cassette arrays, more than one functional transposon version has been identified (Labbate *et al*., 2008; Marchiaro *et al*., 2010). One of these, Tn*6007*, is from a human commensal bacterium and the associated integron has a complete *tni* module that is a hybrid when compared with Tn*402* (Labbate *et al*., 2008). This is a significant observation because it implies either that independent capture events involving the *res* hunter transposons and class 1 integrons can occur or that rearrangements between Tn*402* like integron/transposons and other members of the *res* hunter family are similarly occurring. In either event, it is likely that other analogous variants can be found because the bacterium containing Tn*6007* was recovered in the absence of any selection beyond the ability to grow on complete medium (Labbate *et al*., 2008). One very common feature of class 1 integrons associated with a complete or a partial Tn*402* module is evidence of the presence of a gene conferring resistance to quaternary ammonium compounds. In those class 1 integrons with a 3′-CS, this *qacE* gene has undergone

a deletion, a consequence of the creation of this segment (Stokes & Hall, 1989). In the fully functional class 1 integron/transposons Tn*402* and Tn*6007*, complete *qacE* gene are present as part of mobile gene cassettes (Radstrom *et al.*, 1994; Labbate *et al.*, 2008). Where present, the *qac* genes in such functional class 1 integrons/transposons are in functional cassettes (as distinct from the nonfunctional *qacE* cassette in the 3′-CS); hence, loss of such a cassette is as likely as for a typical antibiotic resistance cassette. Thus, if the progenitor class 1 integron that was captured by the transposition module brought in a *qac* gene, its absence could be explained by cassette deletion. In our view, it is likely that a *qac* gene was present when capture occurred because surveys of class 1 integrons that are embedded in Tn*402*-like transposons very commonly have *qac* cassettes linked to them (Gillings *et al.*, 2009c) as discussed below.

The linking of the class 1 integron to a *res* hunter-type transposon was clearly an important step (Fig. 5) in the introduction of these elements into pathogens, and recent analysis of environmental bacteria has shed some light on how this occurred. When various Proteobacteria from nonclinical environments were tested for the presence of class 1 integrons, it was found that these elements were readily recoverable at a frequency of about 2% of bacteria screened. The study was noteworthy in that bacteria were recovered without selection for antibiotic resistance, the bacteria came from environments that were not under any overt selection for such resistance and the recovery of class 1 integrons was carried out in such a way as to not bias towards association with *res* hunter transposons (Stokes *et al.*, 2006). While some of the integrons recovered were the clinical (i.e. possessed a 3′-CS) Tn*402*-like variants, most were not. This second group was distributed among different Proteobacteria, thereby implying LGT events that were independent of Tn*402*-like transposition systems. Subsequent studies reinforced this point (Gillings *et al.*, 2008), making it clear that class 1 integrons are being mobilized independent of *res* hunter transposons, notwithstanding the prevalence of this type in clinical isolates, and this mobilization probably began well before the beginning of the antibiotic era. The pre-Tn*402*-like integrons commonly possess cassette arrays, although none of the cassette genes are obvious antibiotic resistances genes. Instead, they are more typical of cassettes from chromosomal arrays in the sense of being novel with no close (if any) homologues in the databases. The one exception to this, however, is the common presence of *qac* containing cassettes. In studies based on the 'random' recovery of class 1 integrons from environmental DNA and pure cultures, it was found that over half of the recovered pre-Tn*402* integrons included *qac* cassettes in its array (Gillings *et al.*, 2009c) and that in some communities these cassettes were being actively exchanged (Gillings *et al.*, 2009a). This observation suggests a parallel with mercury-resistant tranpsosons as described above. Specifically, disinfectants predate the clinical use of antibiotics by at least 50 years (Gilbert & Moore, 2005) and quaternary ammonium compounds were a major fraction of these. Given the association of *qac* genes to mobilized, but non-Tn*402*-like class 1 integrons, and the presence of *qac* cassettes in Tn*402*-like integrons that predate the 3′-CS, we argue that selection for *qac* resistance led to at least the partial mobilization of class 1 and amplified their numbers in the Proteobacteria even before the application of the first antibiotics (Gillings *et al.*, 2009c) (Fig. 5). With this scenario, when antibiotics came into broad clinical use, it would be almost inevitable that class 1 integrons would come to play a major role in the dissemination of antibiotic resistance in the same way as mercury-resistant transposons play a similar role.

It is also noteworthy that the debate over the role disinfectant use has in selecting for multi-drug-resistant bacteria is still very much ongoing. As is the case for coselection for antibiotic resistance with mercury, studies also claim that links are lacking between qac and antibiotic coselection (Weber & Rutala, 2006). However, we believe that reductionist studies that look at defined environments over limited time frames are missing real-world events. Given the power of LGT, the antibiotic resistance problem can only be understood and potentially controlled by considering gene flow through the biosphere over time. This point is reinforced by the fact that metagenomic studies, which are culture independent and therefore represent a more inclusive sample of the microbial biosphere, reveal that mobilizing elements like integrons are extraordinarily abundant and that selection in stressed environments with respect to such compounds as heavy metals are enriched with antibiotic resistance genes (Wright *et al.*, 2008; Rosewarne *et al.*, 2010).

The success of the Tn*402*-like class 1 integrons in disseminating antibiotic resistance genes is striking. Other integron classes have been recruited into mobile elements, but their impact has been more limited. The two best examples are class 2 and class 3 integrons. Class 2 integrons were first described around the time of the class 1, when site-specific recombination functions were identified in Tn*7* (Sundstrom *et al.*, 1988). However, this original version, along with most other examples found since, has a nonfunctional DNA integrase via the presence of a premature stop codon in the corresponding gene (Hansson *et al.*, 2002). Not surprisingly, the diversity of the cassette arrays is low compared with class 1 integrons, although some differences can be found (Hansson *et al.*, 2002; Biskri & Mazel, 2003; Plante *et al.*, 2003). This outcome is presumed to be achieved by providing an integrase function *in trans*.

Two functional variants of *intI2* have been found. One is from *Provdentia stuartii* isolates from cattle in Australia

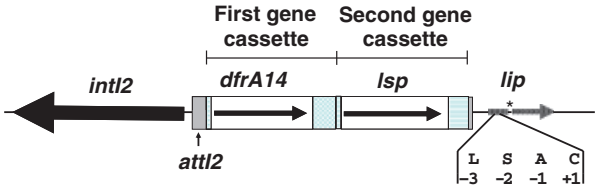

Fig. 6. Structure of functional class 2 integron and associated cassette array from an *Escherichia coli* pathogen. *intI2*, functional class 2 DNA integrase; *attI2*, class 2 integron attachment site; *dfrA14*, trimethoprim resistance gene cassette; *lsp*, putative lipoprotein signal peptidase gene cassette; and *lip*, putative lipoprotein gene. This *lip* gene is not cassette associated. Asterisk indicates the relative position of internal stop codon. Letters indicate a putative signal peptidase cleavage recognition site. The resident strain and other features are as described previously (Marquez *et al.*, 2008a, b).

(Barlow & Gobius, 2006) and the second is from an *E. coli* isolate in Uruguay (Marquez *et al.*, 2008a). Both sets of isolates were additionally noteworthy in that they included gene cassettes that did not possess obvious antibiotic resistance genes. Both these sets of integrons are, or are likely to be, on plasmids and so this may suggest that functional mobile integrons are beginning to recruit other types of genes. The Uruguayan isolate (Fig. 6) highlights the ramifications of this phenomenon for the management of pathogens, because the organism is an actual pathogen and the cassette array carries a known antibiotic resistance gene along with a cassette that includes a gene that encodes a likely lipopolysaccharide signal peptidase (Marquez *et al.*, 2008a). The precise function of this protein is unknown, but bioinformatic analysis strongly suggests a link to a protein family that can impact host pathogenicity. If it is subsequently found that this gene product does have a direct link to virulence, it may be an early portent that integrons in multiresistant strains are beginning to recruit other types of cassettes that enhance pathogenicity. The rules of LGT do not prevent this, because if resistance genes can be recruited, appropriate selection will, by the same rules, see other types of genes appear. Intuitively, if every member of a population is multidrug resistant, the next logical step would be the recruitment of other genes that assist in niche adaptation over competitors. There is at least one other example of an analogous array structure. In a class 1 integron recovered from an *Acinetobacter* from a prawn gut, a two-cassette array was identified (Gillings *et al.*, 2009b). One cassette carried a previously identified resistance gene. The second cassette included an *msr* operon. The *msr* family of genes encodes methionine sulphoxide reductases, multiple copies of which assist in adapting to high-stress environments especially in relation to oxidative stress tolerance in the intestinal tract. This particular integron is from a commensal of an invertebrate that is a popular human food. We consider this link to be a potentially major conduit and we predict that this *msr* cassette will be identified in an integron array in a human pathogen in the near future.

Class 3 integrons were first identified in Japan in 1995 (Arakawa *et al.*, 1995). A later integron survey in Japan suggested that this integron class may be relatively common in that country, although not to the extent of the class 1 (Shibata *et al.*, 2003). The class 3 integron has the potential to spread as it is on a plasmid and has been found in different species within and outside Japan (Correia *et al.*, 2003), but is not common. In systematic screens for mobile integrons, class 3 integrons are rarely detected, suggesting that they have not infiltrated the clinical environments where class 1 (and to a lesser extent class 2 integrons) were common (van Essen-Zandbergen *et al.*, 2007; Laroche *et al.*, 2009). Why is this class relatively rare? One explanation may be the one given above – the class 1 integrons managed to infiltrate clinical isolates in greater numbers first. Another contributing factor, however, may be the fact that the associated class 3 integrase is not as efficient in capturing mobile cassettes as the class 1 in comparative experiments (Collis *et al.*, 2002), thereby potentially conferring another selective advantage on the latter.

Elements within elements

The development of multidrug resistance regions in Gram-negative bacteria in the antibiotic era has been driven by tapping into the vast resource of mobile elements that have evolved in the microbial biosphere over very long periods of time. Some of these preexisting elements have been particularly important in concentrating resistance genes in pathogens. The recruitment of these elements may have been a result of chance or some degree of preselection as described above. In either event, it is clear that only a subset of the available pool of mobile elements has been recruited into pathogens. While small in number, selection has made them extraordinarily abundant. Partly as a consequence of this, drug resistance-carrying mobile elements are cooperating in a way that probably did not occur in the preantibiotic era. Thus, we tend to talk today of multidrug resistance regions and not individual genes. In many cases, the development of multidrug resistance regions has taken place in the chromosome. Several important Gram-negative pathogens are known to have quite variable genomes when different strains are compared and these variable regions, genomic islands, can include concentrations of resistance genes (Dobrindt *et al.*, 2004; Hall, 2010). The expansion of these islands, with ongoing selection, is a major clinical problem in *Acinetobacter baumannii*, where they can extend over several tens of kilobases and comprise dozens of resistance genes (Fournier *et al.*, 2006; Adams *et al.*, 2008). Pathogenicity islands are a form of genomic island that carry known and/or inferred virulence factors. In several organisms, they contribute

substantially to strain-specific pathogenicity and can excise and integrate as a large single unit (Dobrindt *et al.*, 2004). Both genomic islands and pathogenicity islands commonly contain the same types of mobilizing elements seen in extrachromosomal DNA. Genomic islands often include integrated plasmids (Smillie *et al.*, 2010) and the mobilization of pathogenicity islands is mediated by processes similar to that seen for ICEs with respect to incision/excision mechanisms. Indeed, this may be at least partly driven by the integration of whole plasmids or ICEs into the chromosome (Burrus & Waldor, 2004). Movement of genomic islands can occur by conjugation even when conjugation genes are not linked. For example, the conjugal spread of the *Salmonella* genomic island SG1 can be mediated by IncA/C plasmids, specifically those that carry multidrug resistance regions (Douard *et al.*, 2010), providing another remarkable example of mobile genetic regions that 'cooperate' with each other. Other types of large chromosomal regions with properties shared with genomic and pathogenicity islands are also beginning to appear. One possible emerging example of this distinctive genomic resistance module has recently been reported at a defined location in *E. coli* clonal group A (Lescat *et al.*, 2009), which includes determinants conferring resistance to antibiotics, antiseptics and heavy metals.

The extraordinary power of mobile elements to cooperate is commonly seen in the accumulation and concentration of resistance genes into promiscuous plasmids. This cooperation is recent because, while the individual elements that comprise them have existed since before the antibiotic era, they were not seen together. In pathogens, cooperation is the norm and the abundance and myriad of combinations is accelerating the rate of resistance evolution (Walsh, 2006; Marquez *et al.*, 2008b; Garriss *et al.*, 2009). This level of cooperation is remarkable, given that theory would suggest that such cooperation is not a stable evolutionary strategy (Wagner, 2006). However, this is not something that provides a degree of hope in solving the resistance crisis, because strong selection is the driver of this cooperation. Also, game theory makes the point that cooperation is not stable over *evolutionary* periods of time. Thus, even if selection were to suddenly stop, multiresistance regions would persist well beyond time frames relevant to humans. In the meantime, it is inevitable that ever more larger and complex DNA elements will evolve. At least to some extent, this is likely to be driven by the recruitment of new mobilizing elements linking resistance to mobilizable regions in plasmids. There are now several examples of new families of elements, one of which are the IS*CR* elements. These are a group of insertion sequences with similarities, structural and functional, to the IS*91* family. One of their defining features is a process of one-ended transposition that allows the co-option of adjacent sequences (Toleman *et al.*, 2006). This has ramifications for the dissemination of antibiotic resistance because IS*CR* elements are commonly linked to antibiotic resistance genes in pathogens. Although their name did not come until later, they were first observed nearly 20 years ago in clinical isolates (Stokes *et al.*, 1993). However, they appear to have become especially prevalent in recent years and there are now some 19 distinct groups based on sequence differences and there is evidence that, like other types of mobile elements, recombination is beginning to generate novel hybrids (Li *et al.*, 2009b). They are also linked to, or embedded in, other types of mobile elements, with perhaps the best example being the common linkage of IS*CR1* to class 1 integrons (Sohn *et al.*, 2009), where they act cooperatively to mobilize an increasingly diverse range of antibiotic resistance genes.

Another example of resistance recruitment elements are the IS*Ecp1* family of mobile elements. These are mobile and mobilizing elements that contribute to the growing antibiotic resistance problem. These elements are transposon-like in their mode of movement, but are also commonly found adjacent to various resistance genes in a manner structurally similar to the IS*CR* family of elements. Where this linkage is found, the IS*Ecp1* element has the potential to both mobilize and express the linked resistance gene (Karim *et al.*, 2001). IS*Ecp1* elements were first described in the context of the spread of CTX-M family of β-lactamase genes (Karim *et al.*, 2001; Poirel *et al.*, 2003) and numerous examples of linkage of such genes to an IS*Ecp1* element are now known (Canton & Coque, 2006). However, linkage of an IS*Ecp1* to other resistance gene families are also known including to *qnr* (Cattoir *et al.*, 2008), bla_{CMY} (Verdet *et al.*, 2009) and *rmt* (Wachino *et al.*, 2006). IS*Ecp1* regions are commonly linked to other mobile elements such as other transposons, class 1 integrons and IS*CR1* elements so as to generate quite large and complex regions that potentially move as single discrete units (Canton & Coque, 2006; Rice *et al.*, 2008).

Some families of insertion sequences are frequently found in association with antibiotic resistance regions. One of these is IS*26*. Superficially, this is an example of the very common family of insertion sequences, which, like all members of this family, comprises a transposase gene flanked with characteristic inverted repeats. However, in recent years, it has become clear that this insertion sequence is very commonly associated with resistance regions both in plasmids and in chromosomal genomic islands where, when present, it can be often found in multiple copies (Hall, 2007; Doublet *et al.*, 2009; Dawes *et al.*, 2010). The presence of multiple copies of IS*26* probably enhances the mobilization of resistance in a manner directly analogous to that seen for composite transposons. As well, multiple copies can also result in the deletion of resistance and other regions. Thus, IS*26* is driving the evolution of clonal lines at a regional and

global level (Post *et al.*, 2010). Mobilization of resistance regions by IS*26* may not be confined to conventional transposition since it has recently been reported that single IS*26* copies may be able to mobilize adjacent regions, including resistance genes via a circular intermediate (Cain *et al.*, 2010). If so, this would probably be a form of mobilization analogous to the IS*CR* elements. Another family of insertion sequences – the IS*1111* family – is associated with resistance regions and its association with these regions is likely to grow in prominence with time. Members of this family are atypical of transposons in some respects, most notably in that movement does not generate a target site duplication (Partridge & Hall, 2003). Also, these elements target the inverted repeats of other mobile elements including the Tn*21* family of transposons (Partridge & Hall, 2003) and the *attC* sites found in integron-associated mobile gene cassettes (Tetu & Holmes, 2008; Post & Hall, 2009).

Finally, a mobilizing element recently identified in pathogenic bacteria in association with class 1 integrons is the miniature inverted transposable element (MITE). This is a diverse element family with respect to both sequence and properties, with no single set of universal defining characteristics (Delihas, 2008). They do, however, have the potential to move themselves and mobilize other DNA by a nonhomologous recombination mechanism. There are two recent examples where these elements are linked to class 1 integrons and resistance genes. One is from a clinical *Enterobacter cloacae* isolate, where it was shown that when transposition functions were supplied *in trans*, the so-called integron mobilization unit was translocated, with this region including the flanking MITE sequences, the integron and its associated resistance array (Poirel *et al.*, 2009). The second example is from the *msr* cassette containing the strain described above (Gillings *et al.*, 2009b). As noted, this came from an *Acinetobacter* strain that was not a clinical isolate. Also, the two sets of MITE sequences are not identical in sequence, suggesting two independent capture events, although their arrangement with respect to class 1 integrons is similar. We would again suggest that this family of elements will begin to appear in clinical isolates in growing numbers.

Overall, there is a clear ongoing trend towards increasing size and complexity for resistance regions that involves all of the elements described above. These multifactorial interactions are increasing the opportunities for the recruitment of new resistance and other genes into pathogens. Numerous examples of this have been provided, but a further one is offered. In 2009, the recovery of a new metallo-β-lactamase – NDM-1 – was reported from a *Klebsiella pneumoniae* clinical isolate (Yong *et al.*, 2009). Since its first identification, the gene has spread and it has rapidly become a global problem. Even worse is the fact that the gene product inactivates almost all known β-lactam antibiotics and is found in association with other complex multi-drug-resistant regions (Moellering, 2010) such that it is being labelled as contributing to the creation of a new type of superbug (Anonymous, 2010). Examination of the genetic architecture of the resistance regions found in the original isolate (Yong *et al.*, 2009) revealed that the new NDM-1 gene was flanked by a pathogenicity island at one end and a defective IS*26*/Tn*3* region at the other. Other resistance genes/regions were located in association with a class 1 integron (which also included a second novel resistance gene in a cassette), an IS*CR1* element and an IS*EcP1*. All these regions are located on a conjugative plasmid spreading by LGT. This complexity and cooperativity is, thus, now at a point where our ability to identify and characterize these newly emerging mobile regions is testing the limits of sophisticated contemporary clinical and molecular diagnostic tools.

Concluding remarks

What does the future hold for the management of antibiotic resistance? Based on past experience, the future is not promising. Despite the enormous resources devoted to combating and managing the antibiotic resistance problem, positive outcomes are hard to identify, because the problem continues to grow unabated as determined by any measurable metric. There are global increases in the number of multi-drug-resistant nosocomial infections, the number of resistance genes per pathogen, the range of resistant pathogens and the number of infections refractory to antibiotic treatment. Ironically, this arms race is 'funded' by humans through the use and misuse of antibiotics. This is leading to infections that are nearly impossible to treat, and equally concerning, may begin to kill more quickly through the recruitment of other types of virulence factors. Also, the unrestrained growth of the problem has come about despite the fact that the problem has not been ignored. Rather, the literature contains in excess of 200 000 publications devoted to the problem of antibiotic resistance since the 1950s (Davies & Davies, 2010).

To address the problem, we believe that it has to be tackled at a global level. This global approach has to be considered from a number of perspectives. The first of these is that antibiotic resistance is fundamentally an evolutionary problem, and one in which LGT, combined with human-induced selection pressures, virtually makes the entire microbial biosphere into a single evolving community. Both management strategies and basic scientific analysis could benefit by better trans-border coordination. In the case of the former, there has been some progress and to a lesser extent the latter in recent years; however, there is still probably a long way to go. The increasing power of high-throughput technologies also has the potential to analyse mobile DNA at a scale needed to understand the problem

globally and provide the data needed to devise better management strategies. This requires a shift away from a focus on reductionist strategies that analyse the problem from the perspective of specific sets of resistance gene types, specific organisms or specific locations. The need to understand the problem as a global one and, in part, recognize that it is another consequence of globalization, and therefore requires global solutions is recognized by economists (Rudholm, 2002). It is clearly incumbent on microbiologists at the 'coal face' to recognize the same.

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