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REVIEW

Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria

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Bacteria have existed on Earth for three billion years or so and have become adept at protecting themselves against toxic chemicals. Antibiotics have been in clinical use for a little more than 6 decades. That antibiotic resistance is now a major clinical problem all over the world attests to the success and speed of bacterial adaptation.
Mechanisms of antibiotic resistance in bacteria are varied and include target protection, target substitution, antibiotic detoxification and block of intracellular antibiotic accumulation. Acquisition of genes needed to elaborate the various mechanisms is greatly aided by a variety of promiscuous gene transfer systems, such as bacterial conjugative plasmids, transposable elements and integron systems, that move genes from one DNA system to another and from one bacterial cell to another, not necessarily one related to the gene donor. Bacterial plasmids serve as the scaffold on which are assembled arrays of antibiotic resistance genes, by transposition (transposable elements and IS*CR* mediated transposition) and site-specific recombination mechanisms (integron gene cassettes).
The evidence suggests that antibiotic resistance genes in human bacterial pathogens originate from a multitude of bacterial sources, indicating that the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival. In terms of antibiotic resistance, plasmids serve a central role, as the vehicles for resistance gene capture and their subsequent dissemination. These various aspects of bacterial resistance to antibiotics will be explored in this presentation.
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Keywords: antibiotic resistance; plasmid; transposon; integron; resistance gene cassette; IS*CR* element; recombination; conjugation

Abbreviations: CS, common sequence; CR, common region; IS, insertion sequence; RC, rolling circle

Introduction

Post-World War 2 generations born in the developed countries of the world have been highly privileged. Forget the phenomenal increase in affluence over the last 6 decades, put on one side the staggering technological advances and the dubious benefits of email and mobile phones; the immediate post-World War 2 generation was the first to enjoy, from birth, the benefits of a modern health system that has largely abolished the pain and heartache of many infectious diseases, especially those of childhood, that ravaged societies in the past. This was made possible by the discovery and development of a multitude of antibiotics. Directly and indirectly, their use has transformed medical practice to the point where, at least in the developed world, many, if not all, bacterial infections, so long the scourge of humanity, have, until recently, been considered to be little more than a nuisance, rather than the life-threatening conditions they were and can be. However, over the last 10–15 years, this comfortable perception has changed, as illustrated by regular reports in the press, of the rise of 'superbugs' and speculation as to the foreseeable end of the antibiotic era. What has gone wrong in so short a time, little more than two human generations? Are the doom-mongers to be believed, or are the pronouncements of media correspondents largely exaggeration, driven by the desire for a 'good story'? Although antibiotic use clearly has a continuing major and effective role in current medicine, the commentators have also probably got it right in the medium to long term. The ability of bacteria to rapidly evolve into strains that are resistant to antibiotics, although foreseen by Alexander Fleming, has continually been underestimated. We tend to forget that bacteria have inhabited the planet for approximately three and a half billion years, somewhat longer than mankind, and in that time have had to adapt on innumerable occasions to toxic substances suddenly

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introduced into their environments. Indeed, most antibiotics used today have their origins in antibacterial compounds produced by other microbes as weapons with which to protect their territories; that bacteria have not only survived but adapted and proliferated impressively to colonize some of the most inhospitable parts of the planet attests to their powers of adaptation. It should come as no real surprise that they have developed powerful DNA-modifying strategies that greatly facilitate the process of adaptation and, hence, evolution.

Development of resistance to antibiotics by bacteria that threaten human well-being constitutes, arguably, the most serious challenge to the continuing efficacy of much of modern medical practice, such as complex surgical procedures and organ transplants. Antibiotic therapy, as most if not all in the medical profession appreciate, is one of the foundation stones of modern medicine. Without effective procedures to limit bacterial infection, many modern medical procedures would be considerably more risky, if not a complete waste of time and resources, and rates of morbidity and mortality from bacterial infection would be considerably higher than at present. Yet, the ground won in the hard-fought battle for supremacy over these microscopic opponents is in danger of being ceded. Bacteria are not quiescent regarding their fates when faced with annihilation with antibiotics. Over several millennia, bacteria have become adept at dealing with innumerable substances that threaten their survival. For them, antibiotics are just another group of poisonous compounds, the lethal effects of which have to be neutralized in some way. The effectiveness of the various strategies employed is attested by the impressive speed with which resistant versions of human pathogens have emerged to every antibiotic that has been introduced into clinical practice throughout the last six to seven decades. What drives the process? The answer is simple—use of antibiotics and the more the use the greater the likelihood that resistant strains of bacterial pathogens will emerge (Levy, 2002). Obviously, none of the changes that confer antibiotic resistance is acquired by design; rather, changes to the genetic blueprints of all bacteria are made at random. The vast majority make no improvement and are lost from the population over time; however, those that confer advantage are conserved and, given appropriate selection, undergo clonal amplification, a striking example of the Darwinian hypothesis 'survival of the fittest'. Indeed, the whole period of antibiotic use can be considered as one large ongoing experiment designed to test the hypothesis. To date, Darwin's insight has been strikingly confirmed.

Changes can be made to a bacterium's genetic inheritance in two ways; (1) by mutations that alter the pre-existing DNA of the cell—these alterations, base changes and DNA deletions and inversions (Avison and Bennett, 2005), change genes already possessed but do not add new genes, that is new DNA, to the cell genome—and (2) by acquisition of new genetic material, that is capture of genes new to the cell, which expands the genome. This article examines the latter mechanism, which is largely, although not exclusively, responsible for the development of antibiotic-resistant variant strains of bacteria, which cause infections of man and animals.

The phenomenon of gene acquisition implies gene transfer from some outside source; this source is other bacteria. Bacteria have three methods by which DNA may be transferred from one cell to another; transformation, transduction and conjugation. This article will be concerned only with the last—conjugation—and more specifically with the elements that promote it, namely, bacterial plasmids. For more detailed descriptions of transformation, transduction and conjugation see Bennett *et al*. (2004).

Plasmids are the platforms on which gene arrays are assembled and reassorted. The accretion of potentially useful genes on these platforms, promoted by a variety of recombination systems, can permit a bacterial strain to expand its area of operation into niches that were previously denied to it because they were too hazardous, if not lethal. The development of multiple antibiotic resistance is a particularly striking example, which has allowed many bacterial species to gain a tenacious foothold in many of our hospitals, particularly large hospitals where antibiotics are used in large quantities. Accordingly, understanding how antibiotic resistance develops and is spread by mobile genetic elements is a desirable pre-requisite to the design and development of intervention strategies intended to minimize the threat of bacterial infections.

Mobile bacterial genetic elements

Mobile genetic elements fall into two general types; elements that can move from one bacterial cell to another, which in terms of antibiotic resistance includes resistance plasmids and conjugative resistance transposons, and elements that can move from one genetic location to another in the same cell. The latter elements include resistance transposons, gene cassettes and IS*CR*-promoted gene mobilization. Plasmids and conjugative transposons transfer from one cell to another by mechanisms that involve replication. Transposons, gene cassettes and IS*CR*-mediated gene transfer between sites on the same or on different DNA molecules require some form of recombination, which may or may not also include some form of replication (Bennett, 2005). Plasmids accumulate antibiotic resistance genes as a consequence of the activities of at least these three recombination systems.

Bacterial plasmids

The elements that move many bacterial genes from one bacterial cell to another, the so-called horizontal gene transfer, are bacterial plasmids, specifically conjugative plasmids, that is those able to promote their own transfer and the transfer of other plasmids from one bacterial cell to another. Plasmids are best thought of as small, auxiliary, dispensable chromosomes (Figure 1). In general, they exist separately from and are replicated independently of the main bacterial chromosome, although the majority of replication functions are provided by the host cell. They do not accommodate any of the set of core genes needed by the cell for basic growth and multiplication, but rather carry

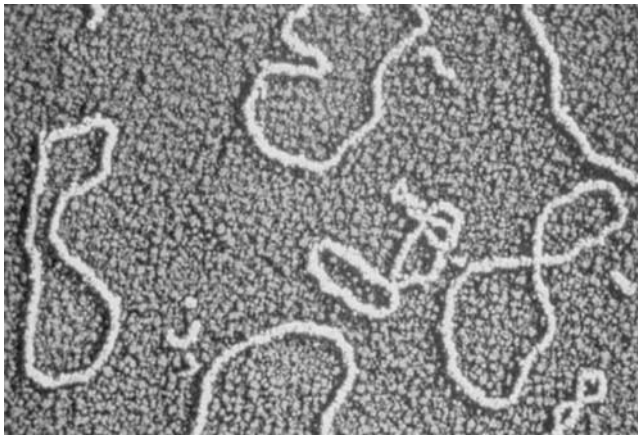

Figure 1 An electron microscope picture of a small bacterial plasmid.

genes that may be useful periodically to enable the cell to exploit particular environmental situations, for example in the current context, survive and thrive in the presence of a potentially lethal antibiotic. Hence, plasmids carry a considerable variety of genes, including those that confer antibiotic resistance and resistance to a number of toxic heavy metals, such as mercury, cadmium and silver, those that provide enzymes that expand the nutritional ability of the cell, virulence determinants that permit invasion of and survival in animal systems and functions that enhance the capacity to repair DNA damage (Stanisich, 1988). Most plasmids investigated so far are circular, double-stranded DNA molecules (Figure 1) that range in size from those with just 2 or 3 genes (2–3 kb) to elements that are equivalent to 10% or more of the host cell chromosome, that is accommodate 400 genes or more (*cf Escherichia coli* chromosome that encodes approximately 4700 genes; Charlebois, 1999). A resistance plasmid is any plasmid that carries one or more antibiotic resistance genes (it may also be, for example, a metabolic plasmid, because it encodes a metabolic function, or a virulence plasmid, because it possesses one or more virulence genes. Carriage of one type of gene does not preclude carriage of other types that do not contribute towards maintenance and spread of the plasmid).

Plasmid-encoded antibiotic resistance encompasses most, if not all classes of antibiotics currently in clinical use and includes resistance to many that are at the forefront of antibiotic therapy. Notable among these are commonly used cephalosporins, fluoroquinolones and aminoglycosides. Many resistance plasmids are conjugative, that is they encode the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer. Others are mobilizable when helped by a conjugative plasmid co-resident in the cell. In general, mobilizable plasmids lack the genes that encode the functions that enable cells to couple prior to DNA transfer (which are provided by the conjugative plasmid) but do encode the functions needed specifically for transfer of their own DNA. Accordingly, mobilizable resistance plasmids tend to be relatively small, often less than 10 kb in size, encoding only a handful of genes including the resistance gene(s), whereas conjugative plasmids tend to be somewhat larger, 30 kb or more (resistance plasmids of 100 kb or more are not unusual), reflecting the sizable amount of DNA (20–30 kb) needed to encode the conjugation functions that permit cell-to-cell coupling, particularly between Gram-negative bacteria. Such coupling is mediated by an external filamentous appendage called a sex pilus, which essentially acts like a grappling hook to join donor and recipient cells and which is then retracted into the donor to effect envelope-to-envelope contact, when a DNA transfer pore forms to bridge the cytoplasmic compartments of the conjoined cells (Wilkins, 1995). Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria, reflecting a somewhat different mechanism of cell-to-cell coupling (see Bennett, 2005), which requires less genetic information. Conjugation is a replicative process that leaves both donor and recipient cells with a copy of the plasmid (Wilkins, 1995).

Conjugative plasmids can exhibit broad or narrow host range. For the latter, transfer is restricted generally to and between a small number of similar bacterial species. Broad host range denotes an element able to transfer between widely different bacterial species and, indeed, some broad host range plasmids from Gram-negative bacteria appear to have no host limitation within the division and, using genetic constructs assembled in the test tube, have been shown to be able to transfer to, but not survive in both Gram-positive bacteria and unicellular eukaryotic microbes such as yeast. One broad host range plasmid is the resistance plasmid RP1 (also known as RP4 and RK4), first identified in a clinical strain of *Pseudomonas aeruginosa*. This plasmid appears to be able to transfer productively to most, if not all Gram-negative bacteria. Many other, unrelated resistance plasmids are also known to have broad host ranges (Thomas, 1989). What determines host range has not been widely investigated, but one possibility is that it reflects the nature of the surface receptor on the potential recipient cell needed by the particular conjugation machinery of the plasmid. If the potential recipient cell lacks this structure, then plasmid transfer to it will not occur. If distribution of the receptor is limited, then the plasmid will exhibit a narrow host range. Another possibility is that although transfer of the plasmid is successful, the recipient cell is unable to support its replication. Both broad host range and narrow host range plasmids are common. Indeed, plasmids are common in most bacterial species investigated to date, identifying a large pool of mobile genetic information. Further, multiple plasmid carriage is not at all uncommon.

Transposons

Resistance transposons are essentially jumping gene systems that incorporate a resistance gene within the element. They come in many forms, distinguished by structure, genetic relatedness and mechanism of transposition and can carry a variety of resistance genes (Bennett, 2005). What is illustrated (Tables 1 and 2) are just a few of those known. All of these elements have the ability to move both intra- and inter-molecularly, that is they can jump from one site to another within a DNA molecule or from one DNA molecule

to another, for example from one plasmid to another, or from a plasmid to a bacterial chromosome and *vice versa*. These mechanisms generally do not require DNA homology between the element and the sites of insertion and although there are examples where a particular transposon has a strong preference for a particular nucleotide sequence at an insertion site, many others show no obvious preference and insert into new sites more or less at random (Craig, 1997).

Transposons belong to the set of mobile elements called transposable elements that encompasses small cryptic elements called insertion sequences (IS elements), transposons and transposing bacteriophages, such as bacteriophage μ (Bennett, 2004). This last type of element is a bacterial virus that uses transposition to replicate. A transposon differs from an IS element in that it encodes at least one function that changes the phenotype of the cell in a predictable fashion, for example a resistance transposon confers resistance to a particular antibiotic(s).

Transposons are either modular systems, referred to as composite transposons, constructed from a pair of IS elements and a central DNA sequence that is not inherently able to transpose, the expression of which alters the cell phenotype (Figures 2 and 3) or complex systems where transposition and non-transposition functions have not obviously been assembled in a modular fashion (Figure 4). For an IS element-dependent resistance transposon, two copies of the same IS element are needed as flanking

Table 1 Some examples of composite resistance transposons from Gram-negative and Gram-positive bacteria

Transposon	*Size (kb)*	*Terminal elements*	*Marker(s)*
Gram-negative elements			
Tn*5*	5.7	IS*50* (IR)	KmBlSm
Tn*9*	2.5	IS*1* (DR)	Cm
Tn*10*	9.3	IS*10* (IR)	Tc
Tn*903*	3.1	IS*903* (IR)	Km
Tn*1525*	4.4	IS*15* (DR)	Km
Tn*2350*	10.4	IS*1* (DR)	Km
Gram-positive elements			
Tn*4001*	4.7	IS*256* (IR)	GmTbKm
Tn*4003*	3.6	IS*257* (DR)	Tm

Resistance phenotypes: Bl, bleomycin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tb, tobramycin; Tc, tetracycline; Tm, trimethoprim.
Abbreviations: bp, base pair; DR, direct repeat; IR, inverted repeat; kb, kilobase.

Table 2 Some examples of complex resistance transposons from Gram-negative and Gram-positive bacteria

Transposon	*Size (kb)*	*Terminal IRs (bp)*	*Marker(s)*
Gram-negative elements			
Tn*1*	*5*	38/38	Ap
Tn*3*	*5*	38/38	Ap
Tn*21*	20	35/38	SmSuHg
Tn*501*	8.2	35/38	Hg
Tn*1721*	11.4	35/38	Tc
Tn*3926*	7.8	36/38	Hg
Gram-positive elements			
Tn*551*	5.3	35	Ery
Tn*917*	5.3	38	Ery
Tn*4451*	6.2	12	Cm

Resistance phenotypes: Ap, ampicillin; Cm, chloramphenicol; Ery, erythromycin; Hg, mercuric ions; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline.
Abbreviations: bp, base pair; IR, inverted repeat; kb, kilobase.

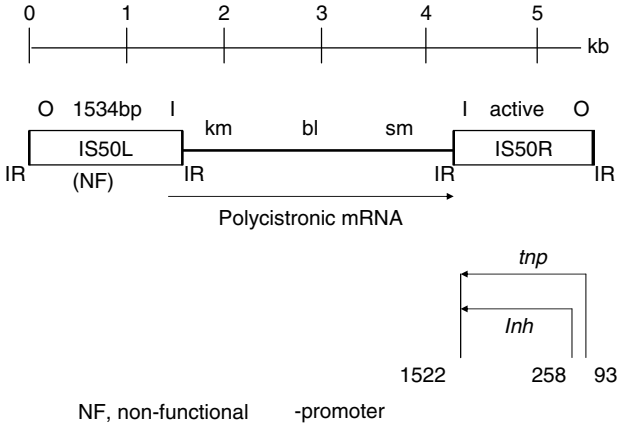

Figure 2 A diagrammatic representation of the composite resistance transposon Tn*5* that confers resistance to kanamycin, bleomycin and streptomycin. O, I, outside and inside boundaries of the terminal inverted IS*50* elements accommodating the short terminal inverted repeats (IRs) that define IS*50L* (left-hand copy of IS*50*) and IS*50R* (right-hand copy of IS*50*) and that are essential for transposition of both IS*50* and Tn*5*; km, kanamycin resistance gene; bl, bleomycin resistance gene; sm, streptomycin resistance gene; *tnp*, gene encoding the IS*50* transposase; *inh*, gene encoding an inhibitor of the IS*50* transposase; bp, base pairs (for further information see Reznikoff, 2002).

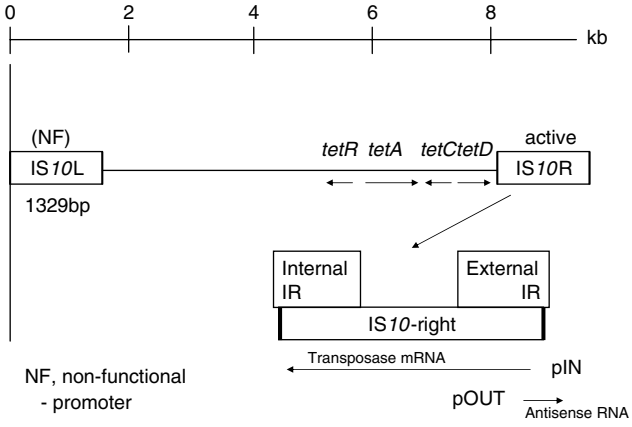

Figure 3 A diagrammatic representation of the composite resistance transposon Tn*10* that confers resistance to tetracyclines. Tn*10* displays terminal inverted repeats of IS*10*; IS*10L*, left-hand copy of IS*10*, a non-functional copy of IS*10* due to multiple mutations in the transposase gene; IS*10R*, right-hand copy of IS*10* that encodes a functional transposase and an antisense RNA molecule used for downregulated expression of the transposase gene; IR, short inverted repeat sequences found at the extremities of IS*10*, which are essential for transposition of both IS*50* and Tn*10*; *tet*A, gene encoding the tetracycline resistance efflux pump; *tet*C,D, genes co-regulated with *tet*A; *tet*R, gene encoding a transcriptional repressor necessary for inducible expression, by tetracycline, of tetracycline efflux pump TetA; bp, base pairs (for further information see Haniford, 2002).

terminal structures, either as direct or inverted repeats, to the central section that contains the gene(s) that confer antibiotic resistance. Although the inverted arrangement of IS elements is more stable genetically, the direct repeat arrangement offers opportunities to migrate to another site where the IS element is also found. This occurs by a two-stage homologous recombination process whereby part of the composite structure is first excised from its existing site by a single crossover between the copies of the IS element releasing a circular, double-stranded DNA species comprising the central section of the composite resistance transposon and one copy of the IS element, the other remaining at the original genetic location. The released DNA can then be rescued, essentially by reversing the first recombination, by homologous recombination involving a single crossover, using the copy of the IS sequence on the free intermediate and another copy at the new location, which recreates the composite transposon at the new site. As IS elements can generally be found at many different sites, particularly on different plasmids, the potential for moving resistance genes around by this method is considerable. Accordingly, there are pros and cons regarding both arrangements. The transposition modules, the IS elements, generally retain the ability to transpose as individual elements as well as part of the compound structure. This is not the case with complex elements, which are indivisible with respect to transposition. The well-known transposons Tn*5*, encoding resistance to aminoglycosides such as kanamycin and neomycin, and Tn*10* encoding resistance to tetracycline, are compound elements found in a number of Gram-negative bacteria, particularly members of the enterobacteriaceae. Such compound structures are created by chance and become established in a population of cells by the selective forces operating on the bacterial flora, for example exposure to kanamycin/neomycin or tetracycline, respectively, when the particular element confers a distinct survival advantage. With time, the structure tends to undergo changes that stabilize it. Many such elements may have a relatively recent genesis. In contrast, Tn*3*, encoding resistance to a number of β-lactam antibiotics, including ampicillin, and Tn*21* (Figure 5), encoding resistance to streptomycin, spectinomycin and sulphonamides as well as mercuric ions, are examples of complex transposons and are also commonly found on plasmids in members of the enterobacteriaceae. Construction of this type of element is less easily explained and no general evidence-based model has yet been proposed, although aspects of construction of some complex transposons, such as Tn*21*, can be deduced. Tn*3*, Tn*21* and similar elements are likely to be of somewhat greater antiquity than most composite transposons and are probably the results of multiple recombination events, including both insertions and deletions, which first insert non-transposition functions into a cryptic element and then refine the sequence by deletion to eliminate 'non-essential' functions. This refinement would make the element more compact and hence more readily transposable. It has been demonstrated that increasing the size of a particular transposable element reduces its frequency of transposition.

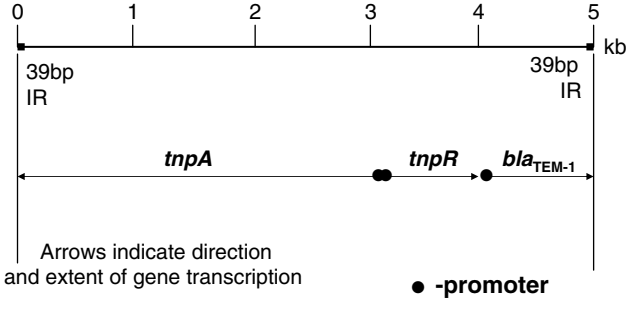

Figure 4 A diagrammatic representation of the complex resistance transposon Tn*3* that confers resistance to ampicillin and some other β-lactam antibiotics. IR, the short inverted repeated sequences found at the extremities of the transposon, which are essential for Tn*3* transposition; *tnp*A, gene encoding element's transposase; *tnp*R, gene encoding a site-specific recombinase that resolves the transposition cointegrate structure generated by transposition; bla_{TEM-1}, gene encoding the TEM-1 β-lactamase; bp, base pairs; kb, kilobase.

Integrons and gene cassettes

Bacterial integrons are gene capture systems that utilize site-specific recombination, instead of transposition, mechanisms (Figure 6). They comprise a specialized recombination system consisting of a gene, *int*, which encodes a site-specific recombination enzyme called an integrase, and a site at which short DNA sequences called gene cassettes (Recchia and Hall, 1995), because most accommodate only a single gene, are inserted by the integrase. In the process of moving from one integron to another, or from one site in an integron to another in the same integron, a gene cassette exists as a small, autonomous non-replicating double-stranded circular DNA molecule. This state is an intermediate in the mechanism that mediates cassette transfer from one

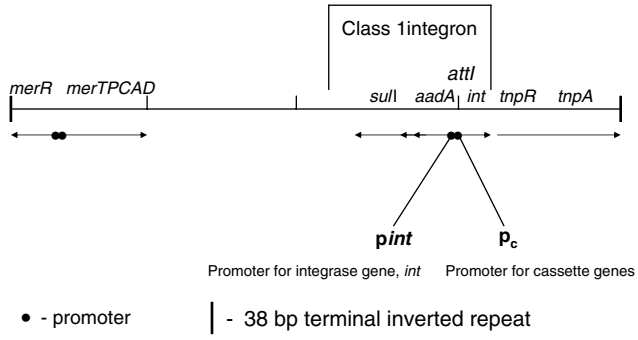

Figure 5 A diagrammatic representation of the complex resistance transposon Tn*21* that confers resistance to streptomycin, spectinomycin, sulphonamides and mercuric ions. *mer*TPCAD, genes encoding resistance to mercuric ions and some organo-mercurial compounds; *mer*R, gene encoding the transcriptional repressor of the inducible mer operon; *sul*1, gene encoding resistance to sulphonamides; *aad*A1, gene encoding resistance to streptomycin and spectinomycin; *int*, integrase gene; *attI*, integron gene cassette insertion site; *tnp*A, gene encoding the Tn*21* transposase; *tnp*R, gene encoding a site-specific recombinase responsible for resolution of the transposition cointegrate structure generated by transposition; p_{int}, *int* promoter; p_c, promoter for integron gene cassettes and *sul*1.

integron to another or the re-assortment of gene cassettes within a particular integron (Bennett, 1999).

Most resistance integrons conform to a structure known as a class 1 integron. Such elements have a distinctive structure comprising two terminal invariable regions, termed constant sequences (CS), and a highly variable central section. At one end is 5′-CS, which accommodates *int*, the cassette insertion site *attI* and the promoter from which cassette genes are expressed. At the other end is 3′-CS, which accommodates part of a gene, *qacE*Δ1, that intact confers resistance to quaternary ammonium compounds, followed by *sul*, a gene that confers resistance to sulphonamides and two other genes designated *orf5* and *orf6*. These CS regions flank a variable one, both in terms of length and sequence, that comprises the gene cassettes of the particular integron. This region necessarily varies as the identities and number of the gene cassettes change from one integron to another.

Resistance gene cassettes lack promoters from which to express the genes they carry. Accordingly, a promoter is provided within 5′-CS for the cassettes that are part of the integron. Necessarily cassette insertion is strictly oriented so that the start of the gene carried by the cassette is the nearest *int*. Gene cassettes can be inserted one after the other into the integron insertion site, *attI* (Figure 7), to produce impressive resistance gene arrays (Figure 8). Each insertion regenerates *attI*. Two or more are expressed in a polycistronic manner from the cassette promoter within 5′-CS and such expression displays polarity. The order of the gene cassettes from 5′-CS indicates the order of addition, the one nearest being the latest addition, because each is inserted at the same point. Accordingly, each new cassette addition displaces the existing array from *attI*. The various resistance cassettes, of which 50–60 are known, confer resistance to several classes of antibiotics (Table 3) (Recchia and Hall, 1995). More recently identified antibiotic resistance gene cassettes include those that encode the metallo-β-lactamases IMP and VIM (Laraki *et al.*, 1999; Lauretti *et al.*, 1999; Poirel *et al.*, 2000; Riccio *et al.*, 2000; Nordmann and Poirel, 2002) (Figure 8), which confer resistance to the potent carbapenem β-lactams imipenem and meropenem. Where individual cassettes originate from is unknown, but impressive chromosomal arrays of gene cassettes encoding a multiplicity of functions, known as super integrons and containing tens of gene cassettes, including resistance gene cassettes, have been found in a number of bacterial species (Rowe-Magnus *et al.*, 2001). The individual cassettes in these arrays can migrate to the much smaller integrons found on plasmids in bacteria of clinical origin, most of which usually contain less than five gene cassettes.

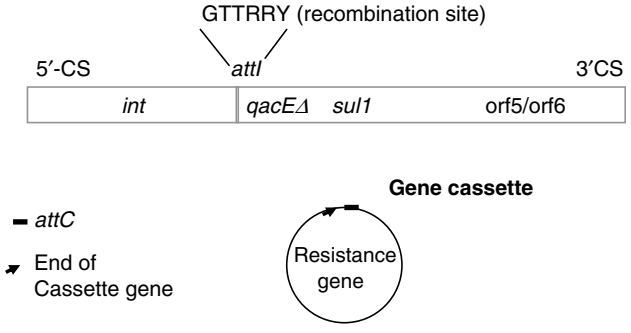

Figure 6 A diagrammatic representation of the elements of a class 1 resistance integron. 5′-CS, 5′ constant sequence; 3′-CS, 3′ constant sequence; *int*, integrase gene; *attI*, integron gene cassette integration site; *att*C, gene cassette insertion sequence (also called a 59 base element); *qac*EΔ, truncated version of the quaternary ammonium compound resistance gene *qac*E; sul1, gene encoding resistance to sulphonamides; *orf*5,6, possible genes (open reading frames) of unknown function(for further information see Hall, 1997).

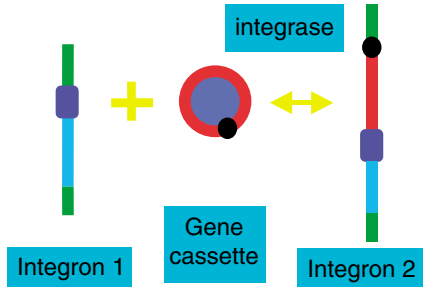

Figure 7 Cartoon of gene cassette capture by a bacterial integron.

IS*CR*-mediated gene transfer

Although the movements of resistance transposons and gene cassettes can account for much of the recombination involved in resistance plasmid construction, it has recently become apparent that there is at least one other recombination system that contributes to the assembly of banks of resistance genes on bacterial plasmids. This aspect of plasmid evolution is based on a set of mobile genetic elements called IS*CR* elements (Toleman, Bennett and Walsh, 2006a). These are small cryptic sequences of sizes similar to those of many IS elements. They are predicted to transpose, like IS elements, hence the IS designation, but by a fundamentally different mechanism called rolling circle (RC) transposition, which couples RC replication, as used by some bacterial plasmids and bacteriophages, and recombination to achieve transposition (Tavakoli *et al.*, 2000; Garcillian-Bracia *et al.*, 2002). IS*CR* elements were first detected as sequences associated with but distinct from class 1 resistance integrons (Figure 9). Because the same sequence was commonly found it became known as the common region, or CR (Stokes *et al.*, 1993). Later it was found that the original CR sequence, now designated as IS*CR1*, is a member of an extended set of similar elements (Figure 10). When the identities of CR sequences were appreciated, it was thought desirable to retain the CR designation for the purpose of continuity, while also advertising their essential nature through use of the prefix IS. To date, more than a dozen related elements have been discovered; now designated as IS*CR1*, IS*CR2* and so on (Toleman *et al.*, 2006a, b).

IS*CR* elements are distantly related to a trio of closely related IS elements, IS*91*, IS*801* and IS*1294* (Garcillan-Bracia *et al.*, 2002; Toleman *et al.*, 2006a, b). It has been known for some time that these elements differ markedly from most other IS elements in both structure and mode of transposition. Most IS elements are delineated by short, inverted

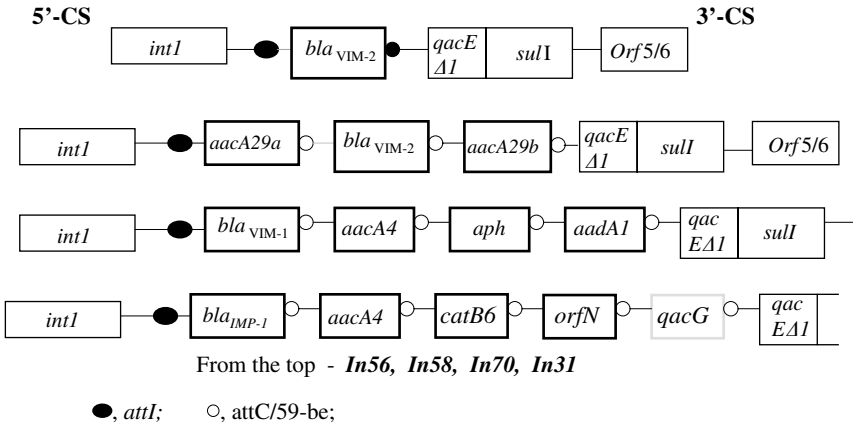

Figure 8 Some examples of resistance gene arrays in class 1 bacterial integrons. *int*1, class 1 integrase gene; *qac*, gene encoding resistance to quaternary ammonium compounds; *att*I, integron gene cassette insertion site; *att*C, gene cassette insertion sequence; *qac*EΔ1, truncated version of *qac*E; sul1, gene conferring resistance to sulphonamides; *orf* (open reading frame), possible gene of unknown function; bla_{VIM}, gene encoding a VIM metallo-β-lactamase; bla_{IMP}, gene encoding an IMP metallo-β-lactamase; *aac*A, *aad*A, *aph*, genes encoding resistance to aminoglycosides; *cat*, gene encoding resistance to chloramphenicol.

Table 3 Examples of resistance genes carried on gene cassettes in class 1 bacterial resistance integrons

Resistance to β-lactams
Class A β-lactamases: *bla* P family (P1,2,3)
Class B β-lactamases:*bla*IMP family (1–8); *bla*VIM family (1,2,3,4)
Class D β-lactamases: *bla*OXA family (1,2,3,5,7,9,10)

Resistance to aminoglycosides
Aminoglycoside adenylyltransferases: *aadA1a, aadA1b, aadA2, aadB*
Aminoglycoside acetyltransferases: *aacA1, aacA4, aacA7,aacC1, aacC*

Resistance to chloramphenicol
Chloramphenicol acetyltransferases: *catB2, catB3, catB5*
Chloramphenicol exporter: *cmlA*

Resistance to trimethoprim
Class A dihydrofolate reductases: *dfrA1, dfrA5, dfrA7, dfrA12, dfrA14*
Class B dihydrofolate reductases: *dfrB1, dfrB2, dfrB3*

Resistance to antiseptics/disinfectant
Quaternary ammonium compound exporter: *qacE, qacG*

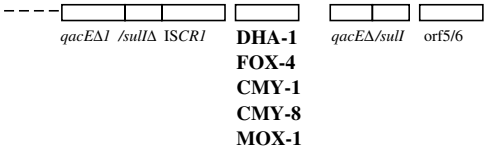

Figure 9 Examples of complex class 1 bacterial resistance integrons displaying copies of IS*CR1* and duplications of 3′-CS. The broken line indicates the 5′-CS and variable regions of the class 1 integron components of the complex integrons; DHA-1, FOX-1, CMY-1,8, MOX-1, genes encoding β-lactamases that confer resistance to third generation cephalosporins, probably recruited from the chromosomes of the bacterial species indicated; for other abbreviations see legend to previous figure.

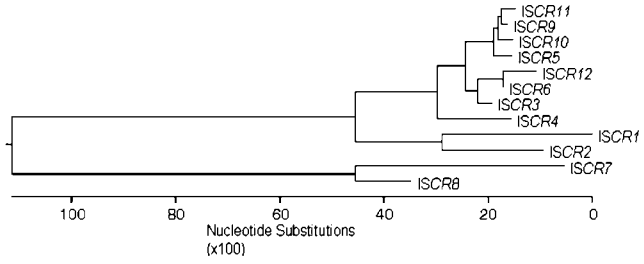

Figure 10 A phylogenetic tree of IS*CR* elements. Based on a CLUSTAL alignment with the PAM 250 matrix prepared using Lasergene DNAstar software (for further information see Toleman *et al.*, 2006a).

nucleotide repeats (see Figures 2 and 3) that functionally are interchangeable and serve to define the ends of the elements and act as binding and cleavage sites for the cognate transposase. Most, if not all, complex transposons also display these features (see Figures 4 and 5). In contrast, IS*CR* elements lack terminal inverted repeats. Rather they possess distinct terminal sequences designated *ori*IS and *ter*IS (see Figure 11) that indicate the unique sites for the initiation and termination, respectively, of the RC replication stage of RC transposition (Tavakoli *et al.*, 2000). One feature of these systems, relevant to the spread of antibiotic resistance genes, is that recognition of *ter*IS shows a degree of inaccuracy, up to 10%, allowing replication to proceed beyond *ter*IS and into the adjacent sequence, where it appears to be terminated more or less at random. Sequences several times the size of the element itself can be co-transposed in this way (Tavakoli *et al.*, 2000), but always those adjacent to *ter*IS. The mobilization is intrinsic to the RC transposition mechanism and will, in principle, mobilize any DNA following the replication termination signal. This is predicted to be the key to the construction of complex class 1 integrons.

Complex class 1 integrons have two notable features; approximately one-half of the structure comprises a typical class 1 integron with 5′-CS, 3′-CS and intervening variable

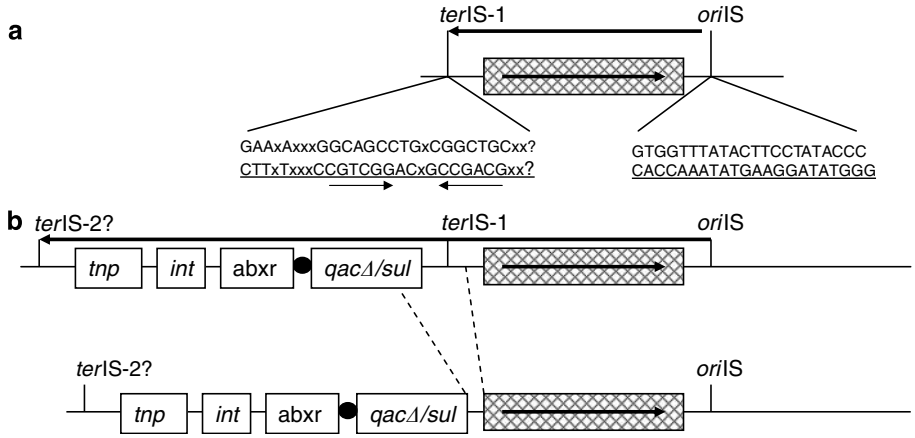

Figure 11 Mobilization of a class 1 integron by IS*CR1*. IS*CR1* is shown in (**a**) delineated by its terminal sequences *ter*IS-1 and *ori*IS. It is proposed that a copy of IS*CR1* is transposed into a site close to the 3′-CS end of a class 1 integron. A deletion then removed part of the 3′-CS (including orf5,*6*) to generate the distinctive 3′-CS-IS*CR1* arrangement seen in complex class 1 integrons.

region, followed by a copy of IS*CR1* and then another variable region that accommodates a variety of resistance genes, including bla_{CMY} and bla_{CTX} variants, *qnr*A, *dfr*A variants and *cat*AII, which between them confer resistance to a range of cephalosporins, fluoroquinolones such as ciprofloxacin, trimethoprim and chloramphenicol. This variable region is, in turn, followed by a repetition of 3′-CS (Figure 9) (Toleman *et al*., 2006b). Accordingly, an explanation of their construction must account for the ubiquitous presence of IS*CR1* and its constant location within 3′-CS, a variety of resistance genes that are not components of gene cassettes and consequently have not been inserted at an *attI* site and the 3′-CS duplication. The model proposed in Figures 11 and 12 does this. It utilizes the known transposition activities of IS*91*-like elements, particularly the tendency to overshot *ter*IS when engaged in RC transposition and the ability to create free circular species, which may be transposition intermediates (Tavakoli *et al*., 2000; Garcillan-Bracia *et al*., 2002).

The model proposes that IS*CR1* became associated with the 3′-CS of a class 1 integron, as a chance event (Figure 11). A series of aberrant transpositions then placed IS*CR1*, together with different lengths of the class 1 integron alongside a variety of resistance genes (Figure 12, step A). From these constructs free, circular species containing IS*CR1*, whole or part of 3′-CS and a non-gene cassette resistance gene(s), were generated by the RC transposition mechanism (Figure 12, step B) and subsequently rescued by homologous recombination into another class 1 integron, using the common 3′-CS sequence as point of crossover (Figure 12, step C). This model accommodates successfully all complex class 1 integron structures identified (Toleman *et al*., 2006a, b) and a number of resistance gene arrays assembled round other IS*CR* elements (Toleman *et al*., 2006a).

For several years, IS*91* and its like were curiosities in the world of IS elements (Garcillan-Bracia *et al*., 2002); a rather esoteric area of study, which was of only minor interest beyond IS aficionados. The discovery of their intimate involvement in the movement of resistance genes that challenge the use of some of the latest and most potent of currently used antibiotics has brought them centre-stage. Further, their ability to mobilize, at least in principle, any bacterial DNA sequence means they pose a considerable threat in the future, their activities being limited only by their distributions. That several, if not all, IS*CR* elements have migrated on to plasmids means that their potential for movement between bacterial species is considerable. Accordingly, a large proportion of the entire bacterial gene pool on the planet is potentially accessible for transposition on to bacterial plasmids. It is to be expected that this is happening all the time, but that only a small minority of such rearrangements are fixed into the populations of cells where they occur because the appropriate selective forces needed to fix and amplify particular rearrangements are missing, at the time. Our intensive use of antibiotics establishes the necessary selective pressures to ensure survival of a particular subset of rearrangements, namely, those involving genes conferring antibiotic resistance to the host cell. Whether these occur in bacteria able to cause infection is immaterial. It is sufficient, in the first instance, simply to preserve and amplify the rearrangement. It will subsequently be spread horizontally by plasmids to other bacteria, among which will be human pathogens. The route may be straightforward or somewhat circuitous, but I have no doubt that the journey will be completed. The only question is 'How long will it take?', a question that cannot be answered with any degree of confidence.

Ramifications of MGE activities

Thus, at least three fundamentally different recombination systems act to assemble and re-assort resistance genes on bacterial plasmids. Any combination may be involved in the generation of a particular resistance plasmid, but the operation of all three mechanisms provides bacteria with an extremely powerful and flexible genetic tool box that makes up with prolific activity what it lacks in direction. It is the difference between a hacker and a computer searching for a password; whereas the former brings to bear intelligence and

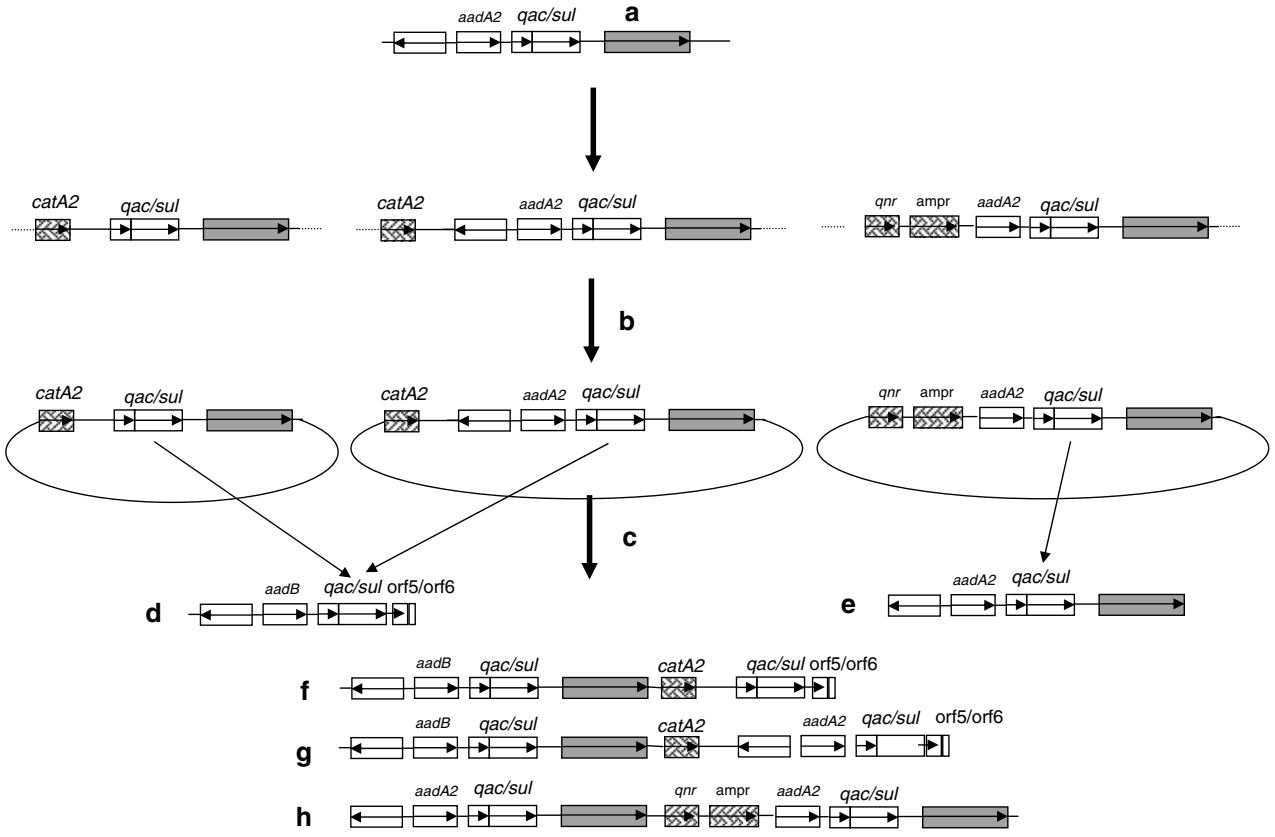

Figure 12 Model of IS*CR1*-mediated generation of a complex class 1 integron. See text for an explanation of the steps involved in generation of complex class 1 integrons.

insight to the task, the latter uses its ability to address all possible permutations. So it is with the global bacterial population. What is lacking in sentient ability is more than compensated for by sheer numbers. One estimate of the global bacterial population is put at 10^{31} individual cells, so one would judge that even if an event occurs only at very low frequency, the sheer size of the global bacterial population means that it is likely to occur and re-occur in many places and on many occasions.

Thus gene cassettes can insert into integrons that comprise part of a transposon that is carried on a plasmid. The integron carrying transposon Tn*21*, found on a plasmid in one of the first multiresistant strains of *E. coli* in the 1960s is a prime example. IS*CR* activity can add further genes to the system via interaction with integron sequences and the whole assembly can then be transferred from one bacterial cell to another. So these various recombination systems provide bacterial cells with the necessary mechanisms with which to capture and re-assort a whole variety of resistance genes that they need now, and in the future. The movement is random, and generally independent of drug use, but drug use provides a powerful selection for these events once they have happened. The consequence is the rise of the 'superbug', bacteria that are largely unaffected by many different antibiotics, a few being resistant to virtually anything in the pharmaceutical armoury that would normally be deployed against them. Notable among them currently are multiresistant strains of *P. aeruginosa* and *Acinetobacter baumannii*. Between them, these recombination systems have the capacity to recruit on to plasmids any gene from any bacterial cell. Thus, as noted above, the entire global bacterial gene pool is, in principle, available as a source of resistance genes. This has major implications for antibiotic therapy. Given that all antibiotics are necessarily selective and none is effective against all bacteria, then for each antibiotic, there is a subset of bacteria that are innately resistant. If the basis of resistance is genetically discrete, that is dependent on a single gene or a few linked genes, rather than the interaction of complex systems, then there is no reason, in principle, why these cannot be mobilized on to plasmids and transferred to other bacteria; witness the somewhat complex genetic make-up of the resistance component of the vancomycin resistance transposon, Tn*1546*, found in the enterococci (Arthur and Courvalin, 1993; Courvalin, 2006). The logical extension of the argument is that, unless an antibiotic acts in a fundamentally different way from those discovered and developed in the past, resistance is likely to arise to any new antibiotic introduced into clinical practice in the future. The only question to be answered is not 'Will resistance arise?' but rather, as noted above, 'When will resistance emerge?'. It is not possible to answer this question. Past experience suggests that clinical resistance will emerge in the short term rather than the long term, that is in 4–5 years rather than decades. The driving force will be the use of the antibiotic and the more effective it is the more it will be used, so 'ratchetting

up' the selective pressure for the emergence of resistant bacteria. Initially this will lead to an increase in the environment of the numbers of innately resistant microbes, most of which are unlikely to cause infection, and a subset with acquired resistance generated by the activities of mobile genetic elements, but their increase in numbers will enhance the supply of potential resistance genes from which bacteria able to cause human infection will recruit those that are suitable for them. Which genes are transferred, how often and to what will be random processes, but some of the results will, in turn, be subject to the selective pressure of exposure to the antibiotic. Some, if not all, of these genetic constructions will survive and will be passed to other bacteria. Eventually, the resistance genes will find their way into bacteria that are intimately associated with man and domestic animals. It is then a short step to their acquisition by potential human pathogens. This is predictable; as noted, the precise time scale is not.

Conclusions

Given that resistance has arisen to all antibiotics introduced into general clinical practice in the past and is likely to arise to any antibiotic introduced in the future, it would be sensible to consider what can be done to minimize the impact of these developments. Modern clinical practice relies on antibiotic use, not only to combat many bacterial infections but also to prevent infection in the first place. If antibiotic use becomes fatally compromised, there may be little we can do when someone is infected apart from palliative care, but thought should be given to what strategies, apart from prophylactic use of antibiotics, can be developed and deployed to avoid bacterial infections.

Despite the high cost of new antibiotic discovery, it is clearly desirable that new antibiotics continue to be developed for clinical use, since the time will come when currently used compounds are compromised by resistance to the point where effective use can no longer be guaranteed. To postpone the emergence of resistance, the level of use of all antibiotics should be reduced to the minimum compatible with clinical imperative. This has to be on a global basis, as resistant strains of bacteria can emerge anywhere and travel rapidly to all parts of the world, courtesy of the world's airlines and mass air travel. Accordingly, world-wide education is needed to emphasize the consequences of overuse and misuse of antibiotics. The emergence of resistance may also be delayed by antibiotic cycling, if this is feasible, or by combination therapy to make treatment more potent; however, the combination of resistance to aminoglycosides and β-lactams is quite common and has probably arisen from the widespread use of aminoglycoside-β-lactam combination therapy.

Given that antibiotic use will lead to the emergence of bacteria resistant to the antibiotic, it is sensible to investigate procedures that will minimize their impact. Accordingly, best practice in hospital infection control systems should be established in all institutions where antibiotics are used routinely, including thorough, regular cleaning and disinfection of hospital wards and surrounds. In the future, this may be facilitated by better design of hospital facilities that minimizes hard-to-access aspects and areas (curves are better than angles) and use materials that clean easily. A science fiction scenario would be the self-cleaning ward. Coupled to this, to ensure bacterial cleanliness, cleaned areas should be subject to bacterial surveillance. Indeed, it may be desirable to subject patients to bacterial surveillance prior to admission to clean areas and to undertake decontamination procedures if contamination is detected prior to admission.

If antibiotic use does become fatally compromised, alternative effective prophylactic procedures will be needed for complex surgical processes, given that it is almost impossible to guarantee aseptic conditions for prolonged recovery periods. One possibility, the development of vaccines that would protect against the most common nosocomial infections, is an obvious suggestion.

Conflict of interest

The authors state no conflict of interest.

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